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G. Seifert (Ed.)

# Oral Pathology

## Actual Diagnostic and Prognostic Aspects

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# Oral Pathology

## Actual Diagnostic and Prognostic Aspects

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# Preface

Oral pathology is a field of pathology which is well established in the United States, in Japan and, within Europe, in the United Kingdom and Scandinavia. In these countries, oral pathology is practised in institutes and departments of oral pathology which are mostly integrated in a special faculty of dentistry, a dental school or a dental institute. In many other countries, oral pathology is integrated as a special field in institutes of general pathology and as part of a faculty of medicine. These different patterns have led to the different development of science and practice in this area. The scientific expansion of oral pathology is documented by the foundation of new scientific societies of oral pathology, such as the International Association of Oral Pathologists (IAOP), and new journals, such as the *Journal of Oral Pathology and Medicine*, *Oral Oncology* (as Part B of the *European Journal of Cancer*) and *Oral Diseases*. Recently, oral pathology has also been recognised as a part of head and neck pathology, an interdisciplinary field of science. Many new methods have been integrated into the scientific work and diagnostic repertoire of oral pathology.

In this volume, a comprehensive review of the importance of new methods in the diagnosis and prognosis of non-malignant disease and malignant tumours in this area is provided. Eight reputed specialists of oral pathology give a concise survey concerning new aspects of diseases of the oral mucosa. The oral mucosa is not only the point of attack in local injuries, but also reflects many systemic diseases of the organism. It is therefore a valuable indicator of diagnosis and prognosis of various pathological processes.

The chapter by D.M. WILLIAMS deals with mucocutaneous conditions affecting the mouth. Most of these diseases are either auto-immune or immunologically mediated. This chapter will review the advances in the understanding of their aetiology and suggests an approach to systematic diagnosis.

The chapter by C. SCULLY gives a survey of the great number and the diversity of oral viral diseases. The past decade has seen intense and increasing interest in the oral health care consequences of viral infection, particularly the possible relationships between viruses and oral diseases, such as malignant neoplasms, the possible infectivity of saliva and oral secretions and, more recently, the oral consequences of infection with the human immunodeficiency viruses (HIV).

The third chapter is a supplement to the previous one and reviews the numerous connections between oral mucosa, acquired immunodeficiency syndrome (AIDS) and oro-facial Kaposi's sarcoma. The diagnostic criteria for both the clinical and histopathological aspects are considered to be of particular importance.

The fourth chapter concerns the new concept of extranodal non-Hodgkin's lymphomas (NHL) of the oral cavity. The head and neck area is the second most common site of extranodal NHL after the gastro-intestinal tract. The importance of identifying the subtle histological features of each, coupled with immunological and molecular biological studies of biopsy tissues, has been discussed.

The fifth chapter focuses on the importance of proliferation markers in oral pathology as indicators of the clinical aggressiveness of human neoplasms. The value of the different methods and their clinical application in the diagnosis and prognosis of oral lesions are compared.

The sixth chapter analyses the special role of the suppressor protein p53 and its occurrence in oral tumours. This contribution focuses on the occurrence of p53 alterations in oral tumours and their significance as well as on the various means currently available for analysis of the p53 gene.

The seventh chapter gives new insights into genomic instability in head and neck cancer. In this chaper, potentially important chromosome regions identified by cytogenetic and loss of heterozygosity analysis in squamous cell carcinomas of the head and neck are reviewed.

The eighth chapter rounds off the volume with a contribution about oncogenes and growth factor receptors as diagnostic and prognostic markers in precancers and cancers of the oral mucosa.

In all the chapters, the most recent methods of immunocytochemistry, in situ hybridisation techniques and molecular pathology are integrated in the classification of the different types of precancerous lesions and cancers, and their value in diagnosis and prognosis is discussed. This volume is an important source of information about new scientific results in the field of oral medicine and pathology. Illustrations, tables and references to the most important literature make this volume a valuable publication for all readers interested in the field of oral medicine.

The editor would like to thank all the authors for their excellent cooperation in completing their manuscripts accurately and carefully; thanks are also due to Mrs. Stephanie Benko of Springer-Verlag, Heidelberg, for her continued support during the planning, preparation and completion of this volume in the present version and to Mrs. Monika Schacht for her excellent work in the extensive correspondence with the authors and the publisher.

Hamburg, May 1996 Gerhard Seifert

# Contents

# Mucocutaneous Conditions Affecting the Mouth

D.M. WILLIAMS



## 1 Introduction

Diseases affecting the oral mucosa are divisible into neoplasms and conditions which are either auto-immune or immunologically mediated. This chapter is prin-

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cipally concerned with those which have an immunological basis, including pemphigus vulgaris, mucous membrane pemphigoid (MMP), linear immunoglobulin A (IgA) bullous dermatosis (LABD), dermatitis herpetiformis, epidermolysis bullosa (EB), erythema multiforme and lichen planus. These may all appear as vesicles, bullae, ulcers and erosions which, in addition to affecting the mouth, may also involve the skin, eyes and other mucosal sites. Accurate diagnosis is often not possible from oral presentation alone, and even systematic clinical evaluation may fail to yield a definitive diagnosis. However, recent advances in understanding the aetiology of a number of these diseases has now made specific, accurate diagnosis possible. Major advances have been made in understanding pemphigus, pemphigoid and the diseases which make up the EB group. This chapter will review the advances in understanding their aetiology and suggest an approach to systematic diagnosis.

## 2 Pemphigus Vulgaris

Pemphigus presents in two main forms: pemphigus vulgaris, which is characterised by low-level intra-epithelial bulla formation, and pemphigus foliaceus, in which epithelial separation occurs at a higher level. Pemphigus vulgaris is far more common in the mouth than pemphigus foliaceus.

### 2.1 Epidemiology

Incidence rates for all forms of pemphigus range between 0.5 and 3.2 per 100000 per annum, affecting both sexes with equal frequency (Korman 1988). It occurs principally in the fifth and sixth decades, although cases have been recorded in children (Ahmed et al. 1980) and adolescents (Ahmed 1983). There is a strong genetic predisposition, and the highest frequency is seen in Ashkenazi Jews (Pisanti et al. 1974).

### 2.2 Oral Manifestations

Pemphigus affects the mouth in up to 70% of cases (Korman 1988), and it is the only site to be affected in over 50% of patients (Ahmed and Salm 1983). Vesicles in the mouth (Fig. 1) rupture extremely early (Correll and Schott 1985), and patients usually present with one or two painful areas of ulceration. Lesions do not heal, leading to extensive oral ulceration (Ahmed et al. 1980). The sites most commonly affected are palate, buccal mucosa and gingivae (Zegarelli and Zegarelli 1977), and lesions may extend directly to the oesophagus (Raque et al. 1970), pharynx (Zegarelli and Zegarelli 1977) and larynx (Ahmed et al. 1980).

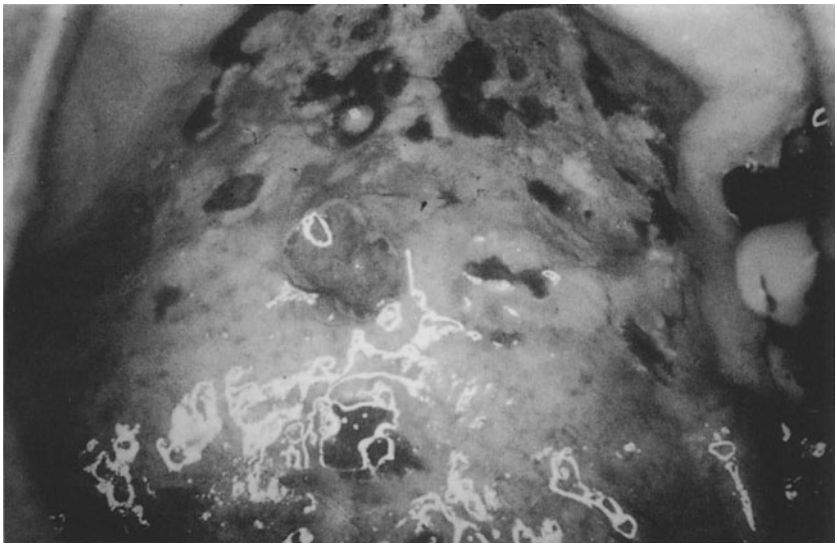

**Fig. 1.** Oral lesions in pemphigus vulgaris. An intact blister on the palate can be seen in the centre, surrounded by areas of ulceration which show no tendency to heal

## 2.3 Skin Lesions

The typical skin lesions in pemphigus vulgaris are fluid-filled blisters which rupture, resulting in areas of denudation. In severe disease, patients may develop electrolyte loss due to the extent of the lesions, together with wound infections, causing management problems. Ocular lesions in pemphigus are usually of minor significance, consisting of a mild, transient conjunctivitis which heals without scarring.

## 2.4 Histopathological Features

Histological examination with immunofluorescence (IF) (BEUTNER and JORDAN 1964; BEUTNER et al. 1965) is crucial in the diagnosis of pemphigus vulgaris. Acantholysis, with separation low in the stratum spinosum, is the diagnostic characteristic of the disease, and this is usually preceded by intercellular oedema and loss of intercellular contacts in the immediately suprabasal cell layers. As separation progresses, bulla formation occurs (Fig. 2a,b), and groups of rounded acantholytic cells, with uniformly hyperchromatic nuclei and homogeneous eosinophilic cytoplasm, are seen floating within the blister fluid. The basal cells remain attached to the basement membrane until the blister ruptures, but secondary inflammation then obscures the diagnostic features. Auto-antibodies directed against an antigen on the surface of stratified squamous epithelial cells are found

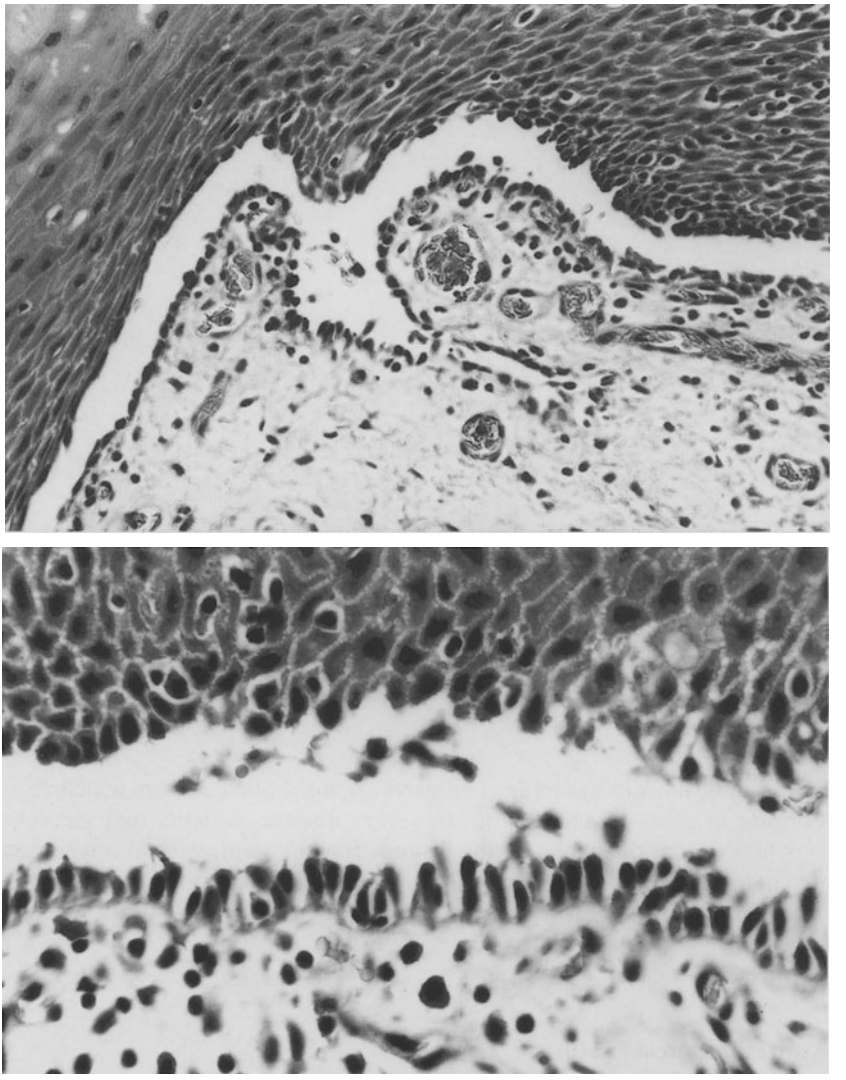

**Fig. 2. a** Medium-power photomicrograph showing the classical features of pemphigus vulgaris. Extensive suprabasal separation has occurred within the epithelium, leaving an intact basal cell layer in the floor of the bulla. There is a striking absence of inflammation in the underlying connective tissue. **b** High-power view of the separation zone in pemphigus vulgaris. The basal cell layer is intact, with separation in the immediate suprabasal layer. Darkly staining acantholytic cells are present, free within the bulla in the centre of the field. The cells in the roof of the bulla also show early features of acantholysis. Their cytoplasm is somewhat shrunken, and there is evidence of intercellular oedema

in pemphigus (Fig. 3), and their presence is the most reliable diagnostic test for the disease. Patients with active disease generally have circulating auto-antibodies, and titres tend to reflect the severity of the disease (FITZPATRICK and NEWCOMER 1980; CRESSWELL et al. 1981).

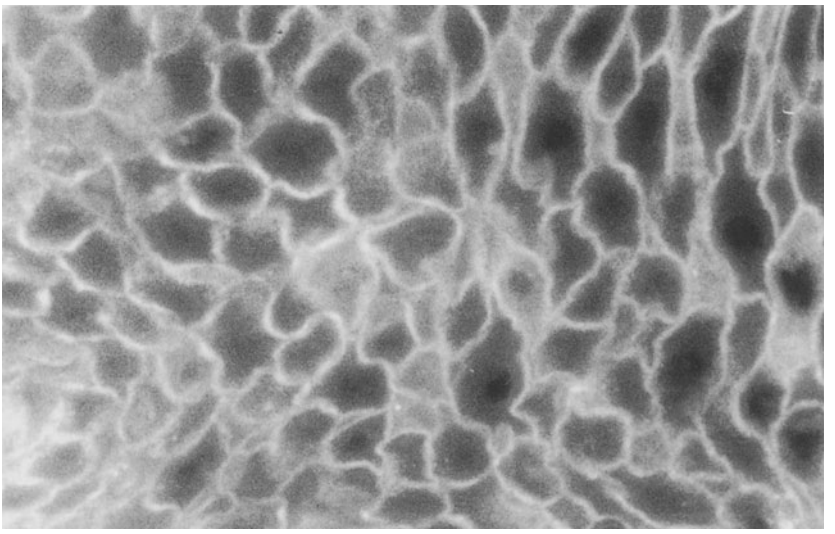

**Fig. 3.** Direct immunofluorescence in pemphigus vulgaris. Binding of autoantibodies to the antigen located in the cell membrane of keratinocytes has produced the fishnet appearance which is diagnostic of pemphigus

## 2.5 Pathogenesis

Recent studies have focused on the molecular basis of normal cell–cell interaction in epithelium and on understanding how interference with this leads to intra-epithelial blister formation. Pemphigus foliaceus is associated with the presence of circulating auto-antibodies to desmoglein-1 (Hashimoto et al. 1990; Calvanico et al. 1991), a transmembrane E cadherin present in all desmosomes. The cadherins are crucial in mediating cell–cell contact, which takes place through their calcium-dependent homophilic interaction (Takeichi 1991). The pemphigus vulgaris antigen (PVA) now appears to be the E cadherin desmoglein-3 (Amagai et al. 1994), which shows significant homology with desmoglein-1 (Amagai et al. 1991). The latter is probably a constant core component of all desmosomes (Koch et al. 1992), whereas PVA seems to be a site-specific desmoglein (Amagai et al. 1991). The different localisation of the site of intra-epithelial blistering and the minor antigenic differences in these two closely related variants of pemphigus reflect heterogeneity in desmosomal structure (Koch et al. 1992) in relation to both site (Ioannides et al. 1991) and stage of differentiation. The production of antibodies against epithelial cadherins interferes with their function as attachment proteins, and the specificity of the auto-antibody then determines the site at which blister formation begins. Once blistering has been initiated, complement- and protease-dependent mechanisms may lead to propagation of the blisters (Williams 1989).

### 2.6 Treatment

The first line of treatment in pemphigus vulgaris is oral prednisolone at a dose of 150 mg per day (Levene 1982), although this dose is increased in severe disease (Eyre and Stanley 1987). Response to treatment is usually rapid and, once the development of new lesions has ceased, medication can be sharply reduced. In some cases, it may be possible to withdraw active treatment, but otherwise low-dose maintenance therapy is indicated.

## 3 Mucous Membrane Pemphigoid

The two principal forms of pemphigoid are bullous pemphigoid and MMP, also called cicatricial pemphigoid. Whilst bullous pemphigoid is essentially a skin disease, MMP mainly affects the oral and genital mucosa and the eyes, with only minor skin involvement. Discrimination between these two diseases on clinical grounds may be difficult, but it is now apparent that there are antigenic differences between them, and this should lead to precise diagnosis in the future.

### 3.1 Epidemiology and Oral Manifestations

The mean age of onset of pemphigoid is 60 years (Bean 1974; Silverman et al. 1986; Venning et al. 1988), with symptoms having a mean duration of 3–5 years (Venning et al. 1988; Silverman et al. 1986). The gingivae are affected in almost all patients (Ahmed and Hombal 1986; Silverman et al. 1986), and the buccal mucosa and palate are involved in between one quarter and one third of patients, with several sites often affected at the same time. MMP typically presents with bullae (Fig. 4), which appear rapidly and then rupture, producing ulcers which heal slowly. Although MMP is often referred to as cicatricial pemphigoid, scarring is not usually a marked feature of oral involvement. MMP is one of the principal causes of desquamative gingivitis (Fig. 5), characterised by the presence of erythematous, shiny lesions confined to tooth-bearing areas (Fine and Weathers 1980). Desquamative gingivitis also occurs in lichen planus, pemphigus vulgaris and psoriasis (Laskaris et al. 1981; Peng et al. 1986), so that further clinical or laboratory investigation is necessary to establish a definitive diagnosis.

### 3.2 Skin and Ocular Lesions

Skin lesions have been reported in up to a third of patients with MMP, and these affect the face, neck and scalp (Person and Rogers 1977; Ahmed and Hombal 1986). However, it is ocular involvement which gives rise to the major problems in

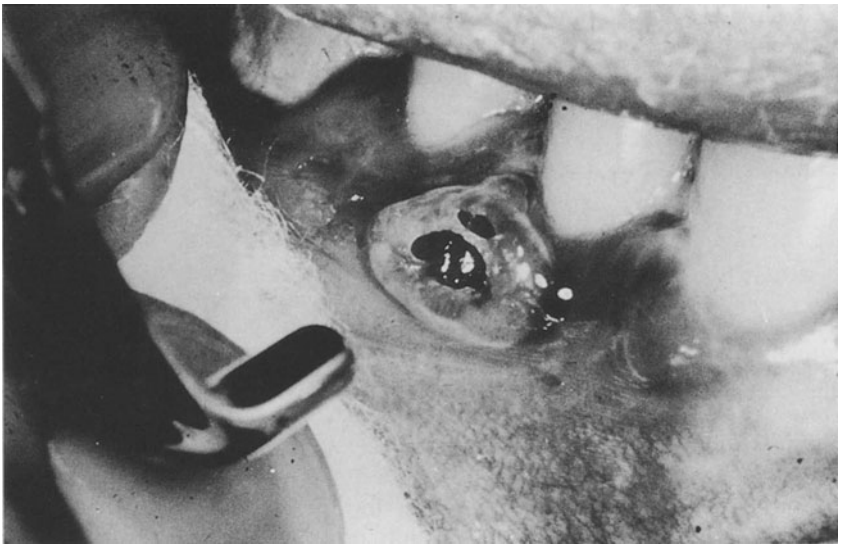

**Fig. 4.** A recently ruptured intra-oral blister in a patient with mucous membrane pemphigoid. A jet of air directed at the lesion has reinflated it

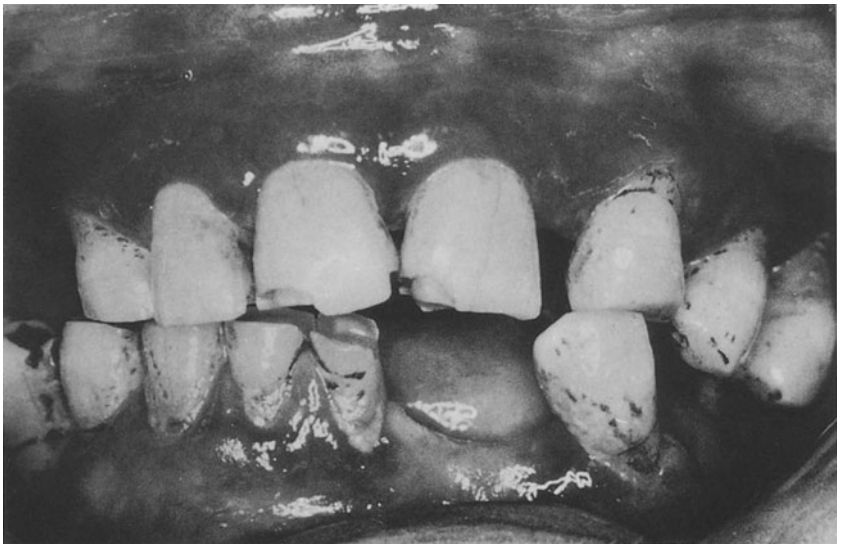

**Fig. 5.** Desquamative gingivitis in a patient with mucous membrane pemphigoid. Bright erythema of the gingivae is seen, confined to tooth-bearing areas. These features are particularly marked in the upper jaw, and the gingivae appear normal in the edentulous lower incisor region

MMP. Patients develop conjunctivitis which progresses to acute ulceration, and blindness occurs in the most severe cases (Person and Rogers 1977; Ahmed and Hombal 1986). Asymptomatic ocular signs have been detected in a high proportion of patients with oral MMP (Williams et al. 1984), and it is recommended that all patients diagnosed as having MMP should receive a thorough, competent, ophthalmological examination.

### 3.3 Histopathological Features

Subepithelial blister formation (Fig. 6) is characteristic of MMP, but it is not unique to the disease. Both bullous pemphigoid and MMP can be discriminated from other clinically similar conditions on the basis of direct IF, which reveals linear binding of Ig to the basement membrane zone (BMZ) at the site of the earliest pathological changes (Fig. 7). Bullous pemphigoid is characterised by linear binding of IgG, usually with C3, to the BMZ, and the majority of patients have circulating anti-BMZ IgG, although antibody titres do not reflect disease severity (Imber et al. 1987).

In MMP, linear BMZ binding of Ig to the oral mucosa and conjunctiva is seen (Person and Rogers 1977; Ahmed and Hombal 1986), but recent studies have reported significant frequency of both IgM and IgA deposition in addition to IgG and C3 (Williams et al. 1984). Attempts to detect circulating auto-antibodies in patients with MMP are usually disappointing using indirect IF, but

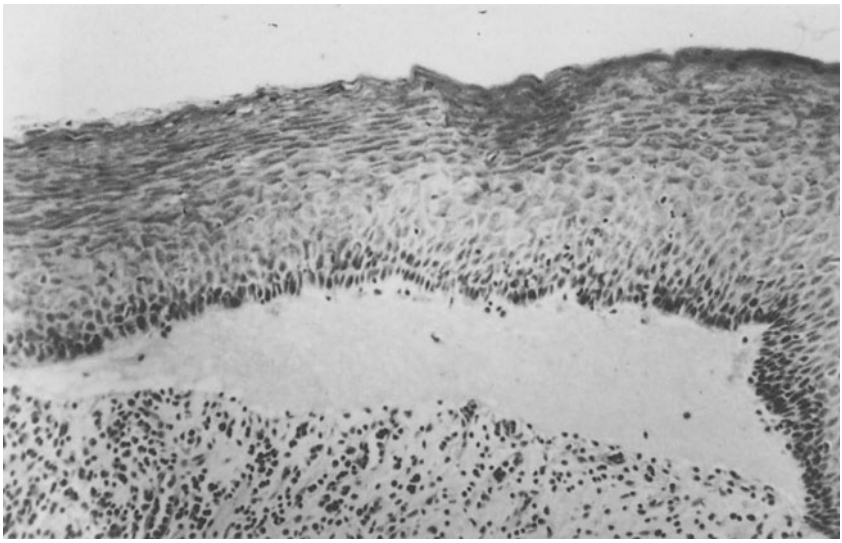

**Fig. 6.** Photomicrograph of an intact bulla in mucous membrane pemphigoid. The bulla is subepithelial in location, and the plane of separation in the basement membrane zone can be seen in the bottom right of the field

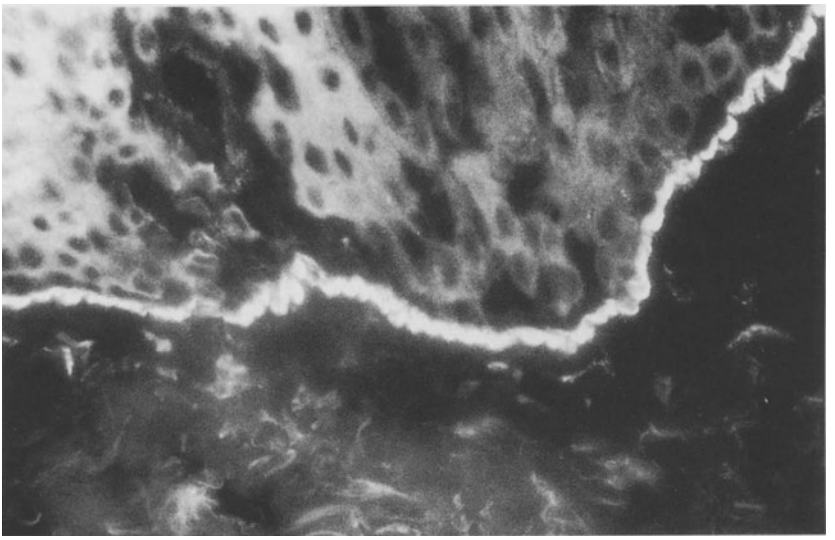

**Fig. 7.** Direct immunofluorescence in mucous membrane pemphigoid, showing bright, linear fluorescence in the basement membrane zone, indicating the site of autoantibody binding

immunoblotting and immunoprecipitation have been claimed to be more sensitive (Bernard et al. 1990; Mutasim et al. 1992).

Interestingly, direct IF performed on clinically normal skin from patients with MMP has revealed linear BMZ Ig deposition in up to 40% of patients (Leonard et al. 1984). Whilst the pathological significance of these observations is uncertain, they may be a reflection of the overlap between MMP and other subepithelial bullous diseases. The presence of IgA in particular raises the possibility of overlap with linear IgA bullous dermatosis (Williams et al. 1984), and precise differentiation of these diseases will depend on identification of the antigen in each case.

## 3.4 Pathogenesis

Just as investigation of the pathogenesis of pemphigus has elucidated the mechanism of cell–cell contact in epithelium, so the study of pemphigoid has revealed the mechanism of epithelial attachment to the basement membrane. Two of the principal components of the basement membrane, which appears ultrastructurally as the lamina lucida and lamina densa, are laminin and type IV collagen (Yurchenco et al. 1992). Attachment of this complex to the deeper connective tissue occurs by anchoring of the fibrils, which are mainly composed of type VII collagen that appears to be of epithelial origin (Regauer et al. 1990). Anchorage of keratinocytes to the basal lamina is mediated via members of the integrin superfamily (Ruoslahti and Pierschbacher 1987) which form stable anchoring com-

plexes (SAC) and focal adhesions (FA). The former are composed of the $\alpha_6\beta_4$-heterodimer and are located at sites of hemidesmosome formation (CARTER et al. 1990a), where they are surrounded by a ring of FA, composed of $\alpha_3\beta_1$-integrin (CARTER et al. 1990b). The ligand for both of these integrins is thought to be an isoform of laminin, known as epiligrin (CARTER et al. 1991; DOMLOGE-HULTSCH et al. 1992), which appears ultrastructurally as the anchoring filaments which traverse the lamina lucida.

In bullous pemphigoid, patients have circulating antibodies against hemidesmosome-associated proteins of 230 and 180kDa, known as bullous pemphigoid antigen-1 (BPAG1) and bullous pemphigoid antigen-2 (BPAG2), respectively (for a review, see WILLIAMS 1990). BPAG1 is located intracellularly and is involved in the linkage between the $\alpha_6\beta_4$-integrin and the intermediate filament system in hemidesmosomes (CARTER et al. 1990a). The antigen has been shown to have substantial sequence homology with desmoplakin (GIUDICE et al. 1992a), which is a component of desmosomes (KOCH et al. 1992). BPAG2, which has been sequenced and cloned (GIUDICE et al. 1992b; LI et al. 1992) and shown to be a 180-kDa hemidesmosome-associated protein (HOPKINSON et al. 1992), has a collagenous extracellular domain and is likely to be involved in cell–matrix interactions. As with antibody binding to desmoglein-3 in pemphigus vulgaris, it is probable that antibody binding with BPAG2 could lead to blister formation in bullous pemphigoid. Whether auto-antibody binding to BPAG1 also leads to blister formation is less clear.

Because patients with MMP generally lack circulating antibodies, precise identification of the antigen has been extremely difficult, although it has been recognised that it is different from the BP antigen (BERNARD et al. 1990, 1992). Indirect immunolectron microscopy has indicated that the MMP antigen is located extracellularly and is probably epiligrin, the key component of anchoring filaments (BEDANE et al. 1991; DOMLOGE-HULTSCH et al. 1992).

### 3.5 Treatment

Although there is no really effective management for MMP (AHMED and HOMBAL 1986), lesions appear to respond to topical steroids (LOZADA-NUR and SILVERMAN 1980). There are anecdotal reports of the use of sulphapyridine and dapsone is severe cases of MMP and in patients who are unresponsive to topical steroids (PERSON and ROGERS 1977; NISENGARD and ROGERS 1987), but there does not appear to have been a well-controlled study of the efficacy of this form of treatment.

## 4 Linear Immunoglobulin A Bullous Dermatosis

LABD is fundamentally a skin disease in which there is spontaneous formation of blisters associated with linear deposition of IgA in the BMZ (WILLIAMS et al. 1984).

Clinical features overlap substantially with those of MMP and dermatitis herpetiformis, with the result that definitive diagnosis depends on laboratory investigation.

Although histopathological examination reveals IgA deposition in the BMZ, this may also be found in MMP, and the antigen against which the antibodies are directed has not yet been identified. However, it appears to differ from that found in both MMP and EB acquisita (EBA) (Wojnarowska et al. 1991), so diagnosis may be made by exclusion.

## 5 Dermatitis Herpetiformis

Although oral mucosal lesions have been described in up to 70% of patients with dermatitis herpetiformis (Fraser et al. 1973), these are not specific, and it is principally a skin disease, with an intensely itchy papulovesicular rash on a background of erythema.

Diagnosis depends on the demonstration of a gluten-sensitive enteropathy and granular deposition of IgA beneath the BMZ in clinically normal skin (Fry and Seah 1974). The mechanism by which IgA becomes bound in the region of the BMZ and the antigen to which it binds are unknown. However, the disease has a strong genetic predisposition (Katz and Strober 1978).

Dermatitis herpetiformis is included here because it must be differentiated clinically from pemphigoid and related conditions and, on histological and IF grounds, from LABD and MMP.

## 6 Epidermolysis Bullosa

### 6.1 Clinical Features

EB is a disease complex comprising at least 20 hereditary and non-hereditary conditions (Wright et al. 1993), all associated with epidermal and mucosal blistering of differing severity (see Table 1). The different forms of the disease are differentiated on the basis of the level at which blistering occurs. These develop within the epithelium in EB simplex, in the BMZ in junctional EB and below the BMZ in dystrophic EB and EBA. Skin and mucous membranes are the primary targets of this group of conditions, although other systems may be involved, and the skin is involved in all of them. The teeth are also affected in some forms of EB.

EB simplex is the most minor of the genetic forms of EB, and blisters generally heal without scarring. Dystrophic EB can occur in dominant and recessive forms and, although both are associated with scarring, this is most severe in the recessive form. Junctional EB may occur in mild and severe forms, and the latter is the most severe of all the forms of EB, due to the fact

**Table 1.** Classification of the hereditary types of epidermolysis bullosa (EB)

| General EB type | Suptype | Mode of inheritance | Distribution of skin lesions | Scarring |
|---|---|---|---|---|
| Simplex | Localised (Weber-Cockayne) | AD | Palms/soles | Rare |
| Simplex | Generalised (Koebner) | AD | Principally extremities | Rare |
| Simplex | Herpetiformis (Dowling-Meara) | AD | Generalised | Variable |
| Simplex | Localised with hypodontia (Kallin) | AR | Hands and feet | Absent |
| Junctional | Generalised (Herlitz, Gravis) | AR | Generalised | Common |
| Junctional | Generalised (Mitis, non-Herlitz) | AR | Generalised | Common but focal |
| Junctional | Localised (minimus) | AR | Hands, feet, pretibial | Absent |
| Dystrophic | Generalised (Pasini, Cockayne-Touraine) | AD | Generalised | Common |
| Dystrophic | Localised (minimus) | AD | Acral | Absent |
| Dystrophic | Generalised (Gravis, Hallopeau-Siemens) | AR | Generalised | Common |
| Dystrophic | Generalised (mitis) | AR | Generalised | Present |

After WRIGHT et al. (1993).
AD, autosomal dominant; AR, autosomal recessive.

that the skin and mucosa become detached from the underlying connective tissue in response to the most minimal trauma. Patients seldom survive beyond the third year of life, with most dying shortly after birth because of overwhelming infections.

EBA is a very different condition from the other genetic diseases in this group. It is a pemphigoid-like disease associated with fragility and blistering of skin and mucosa. This presentation may give rise to confusion with both bullous pemphigoid and MMP on clinical grounds. Furthermore, the histological picture of BMZ separation and the IF appearance of linear IgG and C3 deposition in the BMZ also prevent clear differentiation from pemphigoid (GAMMON et al. 1984).

## 6.2 Oral Involvement in Epidermolysis Bullosa

The extent of oral involvement in EB is variable, depending on the particular form of the disease (see Table 2). In the more minor forms of EB simplex, there may only be a few blisters or vesicles, which heal without scarring. Some degree of oral involvement seems to occur in all forms of junctional and dystrophic EB, but it is particularly associated with the more severe dystrophic forms of EB where it gives rise to major problems of management (NOWAK 1988; WRIGHT et al. 1993). The scarring which occurs on healing results in severe gingival retraction, shallowing

**Table 2.** Oral manifestations in inherited forms of epidermolysis bullosa (EB)

| General EB type | Subtype | Erosions | Scarring | Ankylo-glossia | Micro-stomia | Dental defects |
|---|---|---|---|---|---|---|
| Simplex | Localised (Weber-Cockayne) | Occasional | Absent | Absent | Absent | Absent |
| Simplex | Generalised (Koebner) | Occasional | Absent | Absent | Absent | Absent |
| Simplex | Herpetiformis (Dowling-Meara) | Common | Absent | Absent | Absent | Absent |
| Simplex | Localised with hypodontia (Kallin) | Present? | Absent | Absent | Absent | Absent |
| Junctional | Generalised (Herlitz, Gravis) | Common | Mild/variable | Absent/mild | Moderate | Severe |
| Junctional | Generalised (mitis, non-Herlitz) | Common | Absent/mild | Absent/mild | Absent | Moderate |
| Junctional | Localised (minimus) | Common | Absent | Absent | Absent | Absent/mild |
| Dystrophic | Generalised (Cockayne-Touraine) | Mild/moderate | Absent | Absent | Absent | Absent |
| Dystrophic | Generalised (Gravis, Pasini, Hallopeau-Siemens) | Severe | Severe | Severe | Severe | Absent, except severe caries |

After WRIGHT et al. (1993).

of the vestibule and partial or total tethering of the tongue to the floor of the mouth. Perioral scarring over a period of time causes microstomia, which is particularly severe in recessive dystrophic EB, although it may also occur in the Herlitz variant of junctional EB (WRIGHT et al. 1993).

Involvement of the teeth may be seen in EB, and hypoplastic enamel defects are seen in patients with all forms of junctional EB (WRIGHT et al. 1993), associated with a smooth amelo-dentinal junction. However, there is no consensus on the frequency and extent of dental involvement in all of the EB variants.

## 6.3 Pathogenesis

There have been major advances in understanding EB as a result of clarification of the molecular basis of cell–matrix interaction (UITTO and CHRISTIANO 1992). The Dowling-Meara variant of EB simplex results from structural abnormalities of the keratin filament system (ISHIDA-YAMAMOTO et al. 1991) associated with mutations in the keratin-5 (LANE et al. 1992) and keratin-14 (COULOMBE et al. 1991) genes. Severe recessive dystrophic EB is associated with failure to form normal type VII collagen, leading to abnormalities of the anchoring fibril system (LEIGH et al. 1988; BRUCKNER-TUDERMAN et al. 1989; IWASAKI et al. 1992). The aetiology of the lethal form of junctional EB has recently been clarified with the observation that epiligrin, which is associated with anchoring fibrils, is absent (DOMLOGE-

Hultsch et al. 1992). It is important to recognise, as described in Sect. 3, that the antibodies in MMP are directed at the same protein as is absent in junctional EB.

Antibodies in EBA are directed against part of the type VII collagen molecule (Woodley et al. 1988), the same crucial matrix protein which is absent in hereditary dystrophic EB. Recent comparative IF studies using specific monoclonal antibodies and immunoblotting have shown that the antigen in EBA is probably one of the globular termini of type VII collagen (Woodley et al. 1988). Additionally, it has also been reported that expression of the hemidesmosome-associated $\alpha_6\beta_4$-integrin is normal in EBA, but is altered in bullous pemphigoid (Michalaki et al. 1992). The application of these results to modified diagnostic methods may lead to more reliable differentiation in the future.

## 7 Erythema Multiforme

Erythema multiforme is a mucocutaneous disease predominantly affecting young adults. The oral and skin manifestations vary both in severity and appearance. Attacks are usually of acute onset and may be isolated or recurrent. There are two principal forms of the disease, namely erythema multiforme minor, which is more common, and erythema multiforme major (Brice et al. 1990). The latter is a bullous form of the disease, often termed Stevens-Johnson Syndrome, which is characterised by severe mucosal involvement. As its name implies, erythema multiforme minor is less severe, with relatively minor oral symptoms. It is becoming apparent that these two forms of the disease may be different conditions, with erythema multiforme minor being the more common.

### 7.1 Oral Manifestations

It has been reported that approximately 25% of patients with typical erythema multiforme minor have oral lesions (Brice et al. 1990), although our own observations indicate that they are significantly more common (Farthing et al. 1995). The tongue, buccal mucosa and lips are the most commonly affected oral sites (Lozada-Nur et al. 1989), but gingival involvement is also seen. Patients typically present with lesions that are rather non-specific discrete erosions (Fig. 8) with an erythematous margin, although gingival lesions may be more extensive and confluent.

The mouth is usually involved in Stevens-Johnson syndrome, or erythema multiforme major, which is characterised by severe involvement of at least two mucosal sites (Brice et al. 1990). In classical cases, large, fluid-filled blisters develop rapidly on the lips (Fig. 9), but these usually burst within a day (Fig. 10) to produce crusted lesions which can persist for up to 4 weeks. In the case of severe oral involvement, eating can prove extemely uncomfortable and difficult, and patients may require hospitalisation for supportive therapy.

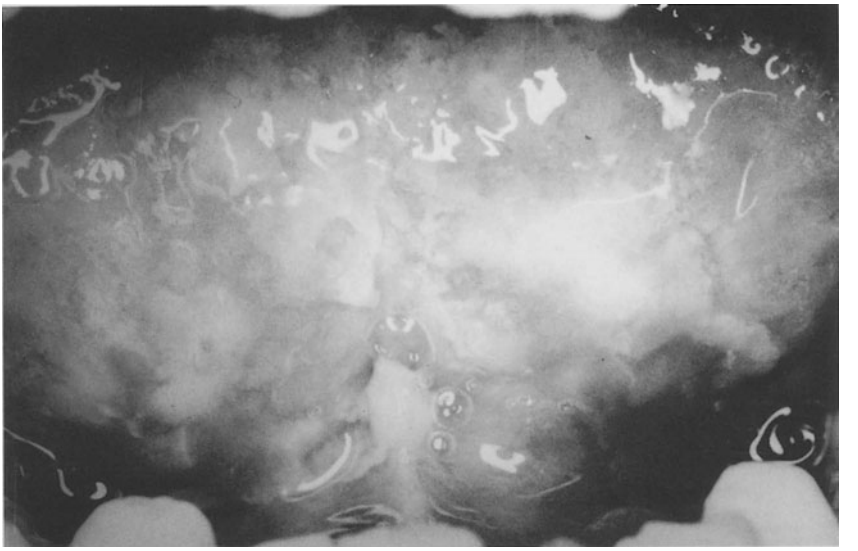

**Fig. 8.** Lesions of erythema multiforme on the ventral surface of the tongue. These comprise extensive, non-specific, sloughing erosions. The appearances seen here are not sufficiently characteristic to permit diagnosis without further clinical information

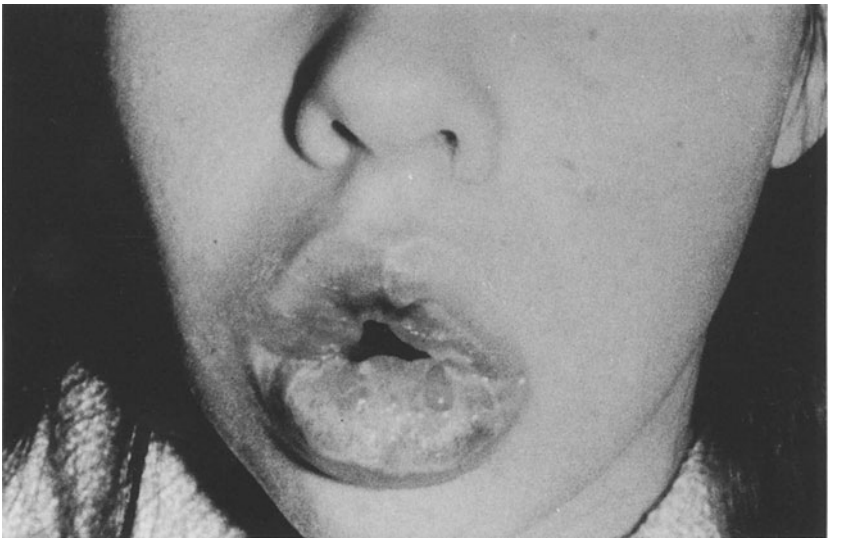

**Fig. 9.** Large fluid-filled blisters on the lips of a 10-year-old girl with Stevens-Johnson syndrome. The blisters do not extend beyond the vermilion border of the lips

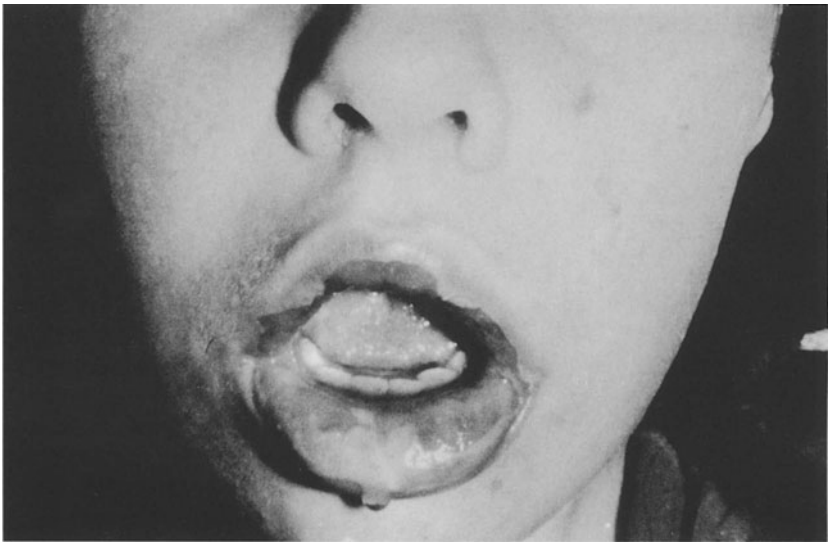

**Fig. 10.** The same patient as in Fig. 9, 24 h later; the blisters have burst, leaving extensive areas of ulceration which subsequently became encrusted

Important insights into the aetiology of erythema multiforme have come from the observation that, although it can occur as a single attack, it is recurrent and has a strong association with herpes simplex virus (HSV) infections, episodes often being preceded by herpes labialis.

## 7.2 Skin Lesions

The onset of erythema multiforme may be preceded by prodromal symptoms, which tend to be more frequent and more marked in erythema multiforme major. Patients who experience frequent recurrences often report discomfort at the site where lesions subsequently develop approximately 24 h before the appearance of physical signs (D.M. WILLIAMS and I.M. LEIGH, unpublished observation). The cutaneous characteristics of both erythema multiforme major and minor have been reviewed by BRICE et al. (1990). Erythematous macules which expand, giving rise to typical "iris" or "target" lesions, with a central dusky zone surrounded by an erythematous outer zone, are the first lesions to appear in both forms of the condition. In some sites, blood-filled blisters may develop in the central area. Individual lesions subsequently expand and merge to produce extensive, irregularly shaped areas. Lesions are often bilaterally symmetrical, affecting the hands, feet, arms and legs, but the basis of this pattern of distribution is unknown.

The lesions of erythema multiforme minor generally heal within 3 weeks, although some patients with continuously recurrent erythema multiforme develop new lesions before those from a previous episode have healed. Both the oral and

cutaneous lesions of erythema multiforme major tend to be more extensive and long-lasting than in the minor form of the condition.

## 7.3 Histopathological Features

Virtually all studies on the histopathological features of erythema multiforme are based on biopsies of established clinical lesions, and the earliest stages in the process have gone largely unreported. However, if patients with recurrent erythema multiforme are biopsied in the prodromal phase, before there is clinical evidence of a lesion, distinctive histological changes can be seen (D.M. WILLIAMS, I.M. LEIGH and P.M. FARTHING, unpublished observations), which point to the conclusion that the keratinocyte is the initial target for cell-mediated immunological damage (MARGOLIS et al. 1983; ZAIM et al. 1987).

We have observed that Langerhans cells migrate from their customary suprabasal position in the epithelium to the upper cell layers, associated with a reduction in the length of dendritic processes. This is accompanied by intercellular oedema within the epithelium and the accumulation of helper/inducer and suppressor/cytotoxic T cells in the epithelium and adjacent connective tissue. With the progressive increase in the lymphocytic infiltrate, keratinocyte damage becomes apparent, followed by necrosis of the epithelium and sloughing. It is at this relatively advanced stage of lesion development in histological terms that the lesion is evident clinically.

An additional feature of the established lesion, as the influx of inflammatory cells in and around the epithelium progresses, is marked perivascular lymphocyte accumulation. Given that most earlier studies were of well-developed lesions, it is not suprising that attention focused on the importance of a vasculitic process in the aetiology of erythema multiforme (TONNESON et al. 1983). Circulating immune complexes can be detected in a significant proportion of patients with recurrent erythema multiforme (LEIGH et al. 1985), and direct immunofluorescence often reveals complement activation in the walls of blood vessels in early lesions (D.M. WILLIAMS, unpublished observation). However, the significance of complement activation is unclear, and the perivascular accumulation of lymphocytes is not specific to erythema multiforme.

With the progression of lesion development, keratinocyte damage becomes marked, with the influx of a mixed inflammatory infiltrate containing significant numbers of polymorphonuclear leukocytes (PMN). This is accompanied by bulla formation and sloughing of the epithelium. By this stage, the histological features are non-specific, and diagnosis is made on the basis of the clinical features.

## 7.4 Pathogenesis

A large number of agents have been implicated in the etiology of erythema multiforme (for a review, see BRICE et al. 1990):

1. Herpes simplex virus
   a) Especially in recurrent erythema multiforme minor
   b) HLA-related susceptibility
2. Ultraviolet (UV) light – associated with HSV activation
3. Drugs – especially in erythema multiforme major
4. Foodstuffs
5. Unknown

Drugs have been particularly associated with the triggering of Stevens-Johnson syndrome (GEBEL and HORNSTEIN 1984), leading to the view that allergic mechanisms might be implicated in the pathogenesis of lesions. This conclusion was supported by the finding of circulating immune complexes and complement deposition in intralesional blood vessels, as described above. More recently, attention has shifted to the keratinocyte as the principal target in erythema multiforme, with increasing evidence for the role of HSV infection in the pathogenesis of lesions (BRICE et al. 1989; HUFF and WESTON 1989; ASLANZADEH et al. 1992; MIURA et al. 1992). HSV DNA has been identified both in the active (BRICE et al. 1989; ASLANZADEH 1992) and healed (MIURA et al. 1992) lesions of erythema multiforme in patients who develop recurrent disease following episodes of herpes labialis. The role of HSV DNA in lesion development is further supported by the observation that prophylactic acyclovir therapy can suppress recurrent erythema multiforme (MOLIN 1987). Whilst there is accumulating evidence that keratinocyte infection by HSV triggers the cycle of lymphocyte-mediated damage, as described above, the mechanism by which HSV DNA becomes localised to keratinocytes, and why this should lead to erythema multiforme, remains to be established. Interestingly, an increased likelihood of developing erythema multiforme following HSV infection has been noted in patients with HLA DQw3 (KAMPGEN et al. 1988), which raises the possibility that the linkage of the two conditions is related to genetic factors. This matter merits further investigation.

The pathogenic mechanisms involved in patients whose disease is triggered by drugs or certain foodstuffs (LEWIS et al. 1989) remains largely speculative.

## 7.5 Treatment

Recognition of the association between HSV infection and recurrent erythema multiforme minor has had a major impact on treatment of the condition. Treatment with acyclovir during the prodromal stage has led to attacks either being aborted or markedly reduced in severity. Prophylactic acyclovir therapy is indicated in patients with severe or continuous recurrent erythema multiforme, and in particularly intractable cases azathioprine may also be required to supplement antiviral treatment (FARTHING et al. 1995). Symptomatic supportive treatment, including mouthwashes for mucosal involvement, may

be indicated. The treatment of erythema multiforme can be summarised as follows:

- Acyclovir, supplemented with azathioprine in severe cases
- Supportive therapy
- Mouthwashes
- Prevention (prophylactic acyclovir, avoidance of triggers)

Patients with erythema multiforme major (Stevens-Johnson syndrome) suffer from more severe systemic upset than those with erythema multiforme minor. Intensive supportive treatment may be necessary, if mucosal lesions interfere with proper nutrition and skin involvement is sufficiently severe to raise the possibility of electrolyte loss and wound infection developing.

## 8 Lichen Planus

Although lichen planus is not a classic auto-immune disease characterised by the presence of circulating auto-antibodies, there is good evidence that it is immunologically mediated. An important clinical aspect of the disease is that it often causes desquamative gingivitis, which is clinically indistinguishable from that seen in MMP.

### 8.1 Epidemiology

A specific antigen has not been identified in lichen planus, and the disease usually develops spontaneously, although it may be precipitated by trauma, such as periodontal surgery (Katz et al. 1988), and by a wide range of drugs (for a review, see Scully and El-Kom 1985). The clinical features are strikingly similar to those seen in graft versus host disease (Jungell 1991), supporting the view that the disease results from cell-mediated immune response against an intra-epithelial antigen.

Lichen planus is one of the commonest mucocutaneous diseases, affecting 1% of the general population. It is generally a disease of the middle-aged (Thorn et al. 1988), although atypical cases have been reported in children (Milligan and Graham-Brown 1988).

There is an established risk of malignant transformation associated with oral lichen planus, with frequency rates between 0.4% and 12.3% (Holmstrup et al. 1988). In their study of 611 Danish patients with lichen planus followed up for periods of up to 26 years, Holmstrup et al. (1988) observed significantly more cases of oral cancer than would have been predicted by chance. It remains a matter of debate as to whether the lesions themselves are precancerous or whether mucosa affected by the disease is more susceptible to the effects of other carcinogens,

but the practical management conclusion is that patients with lichen planus require long-term follow-up.

## 8.2 Oral Manifestations

There are six recognisable forms of lichen planus: (1) reticular or "classic", (2) papular, (3) plaque-like, (4) atrophic, (5) erosive or ulcerative and (6) bullous. Desquamative gingivitis is the result of gingival involvement in erosive lichen planus. In an extensive study of oral lesions in lichen planus, Thorn et al. (1988) found that reticular lichen planus (Fig. 11) was the most common presentation of the disease, affecting over 90% of patients. Several forms of the disease were often found to coexist, with involvement of more than one site. Although site frequency was not reported in the paper by Thorn et al. (1988), one third of their patients had gingival lesions (P. Holmstrup, personal communication) at initial presentation, and a third of those showed remission of their gingival lesions during the course of the study. In contrast to cutaneous lichen planus, a prolonged clinical course is typical of the oral lesions and, whilst individual lesions do not remain static, oral mucosal involvement may persist for many years. Erosive lesions are the most persistent, and they also represent the most common gingival form of lichen planus, with extensive involvement of the attached gingiva in tooth-bearing areas giving rise to desquamative gingivitis. The lesions appear fiery red and are sore. As stated earlier, this clinical appearance may be indistinguishable from the

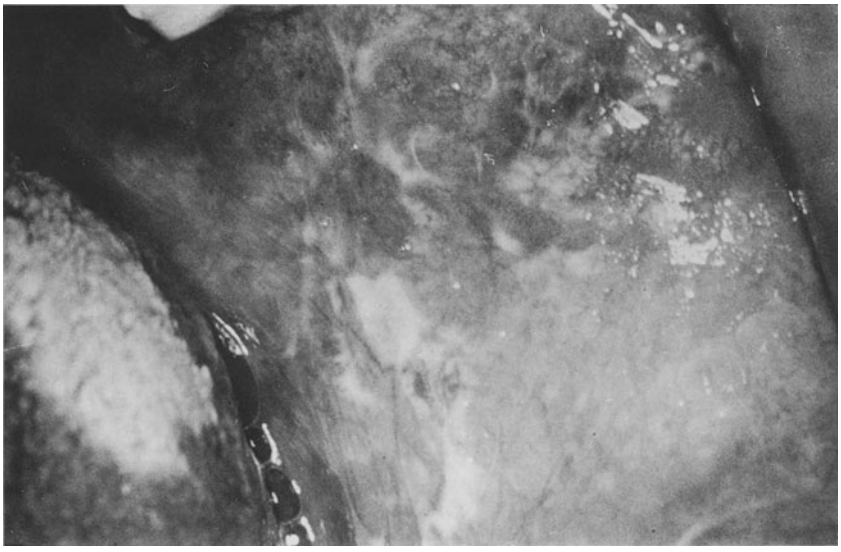

**Fig. 11.** Clinical photograph of classical reticular lichen planus involving the buccal mucosa. Lesions appear as white striations on an erythematous background, giving rise to a lace-like pattern of hyperkeratosis

desquamative gingivitis seen in MMP and, although careful clinical examination often reveals typical lesions of lichen planus at other sites, a biopsy and IF may be required for a definitive diagnosis.

## 8.3 Skin Lesions

The skin lesions in lichen planus typically appear as flat-topped violaceous papules, distributed in groups and principally affecting the flexor aspects of the wrists, the anterior surface of the lower legs, the lumbar region and the genitalia. Scratching or similar trauma may trigger the appearance of skin lesions, a characteristic termed the Koebner phenomenon, and this may lead to a linear clustering of the typical papules.

The skin lesions of lichen planus usually clear within 2 years (SCULLY and EL-KOM 1985) and have no reported malignant potential. Skin and oral lesions may co-exist, and around half of the patients with cutaneous lichen planus also have oral lesions. However, about one quarter have only oral lesions (JUNGELL 1991), which may be a reflection of the more prolonged clinical course of oral lesions than those affecting the skin.

## 8.4 Histopathological Features

Patients with lichen planus typically present with hyperkeratotic but atrophic epithelium and a dense lymphocytic infiltrate in the underlying connective tissue (Fig. 12). The inflammatory infiltrate is restricted to the upper corium, with an abrupt cut-off on its deep aspect, and the adjacent epithelium exhibits basal cell degeneration with an eosinophilic coagulum in the basement membrane associated with lymphocytic infiltration of the epithelium. The histopathological features of lichen planus can be summarised as follows:

- Epithelial atrophy with hyperkeratosis
- Loss of basal cell layer
- Civatte or colloid bodies
- Dense lymphocytic accumulation in upper dermis adjacent to epithelium

One of the shortcomings of many of the histopathological studies of lichen planus is that the amount of clinical information recorded is generally insufficient to establish the duration of lesions. Thus the results from investigations of "young" and "old" lesions have not been differentiated, and it is difficult to establish the pattern of evolution of the disease. It appears from immunocytochemical studies (FARTHING et al. 1990) that there is clustering of Langherhans cells within the epithelium associated with an increase in the number of intra-epithelial lymphocytes and with the accumulation of lymphocytes in the adjacent lamina propria. It is striking that the areas of hyperkeratosis are closely associated with the infiltration of the lower epithelial layers by lymphocytes. Hyalinised, eosinophilic Civatte

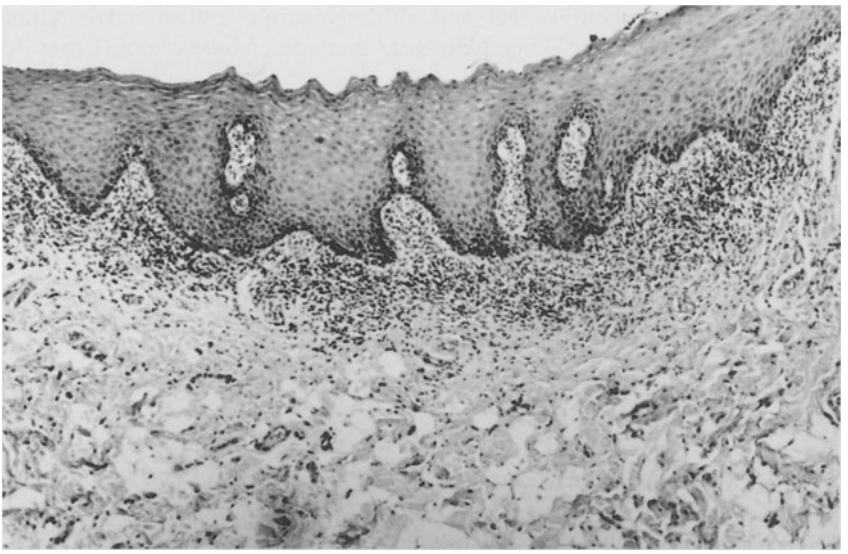

**Fig. 12.** Medium-power photomicrograph showing the typical features of lichen planus of the buccal mucosa. The epithelium exhibits a tendency to atrophy and is parakeratinised. A dense lymphocytic infiltrate is present in the upper part of the corium, with an abrupt cut-off on its deep aspect

bodies are occasionally present within the epithelium, and these are generally believed to be degenerate keratinocytes.

IF in lichen planus does not show characteristic features of the kind seen in either pemphigus or pemphigoid, but direct IF occasionally reveals accumulations of fibrin at the basement membrane zone, and Ig binding is associated with Civatte bodies (Scully and El-Kom 1985).

## 8.5 Pathogenic Mechanisms

Although there has been a substantial amount of research into the pathogenic mechanisms involved in lichen planus, the mechanism by which keratinocyte damage is initiated remains obscure, and no lichen planus-specific antigen has been identified. Nevertheless, the processes by which lesions develop and persist have begun to emerge (Farthing et al. 1990; Walsh et al. 1990; Hedberg and Hunter 1987; Rich and Reade 1989; Boisnic et al. 1990; Farthing and Cruchley 1989):

- Clustering and activation of Langerhans cells in epithelium.
- Keratinocytes express HLA-DR and intercellular adhesion molecule (ICAM)-1.
- Selective accumulation of cytotoxic/suppressor T cells within epithelium.
- Helper/inducer T cells persist in dermis.

- T cells are memory cells in both sites.
- Upregulation of ICAM-1 and E selectin expression on endothelial cells.

As described in Sect. 8.4, focal accumulations of Langerhans cells occur within the epithelium, expressing increased levels of HLA-DP and HLA-DQ (FARTHING et al. 1990), indicating that they are activated. The lymphocytes in the lamina propria are predominantly of the helper/inducer phenotype, but the lymphocytic infiltrate in the epithelium is the result of selective migration of cytotoxic/suppressor cells, which appear to accumulate in areas where there is selective expression of HLA-DR and ICAM-1 on keratinocytes. The lymphocytes in both epithelium and lamina propria are memory T cells, indicating that they are responding to a previously encountered or persistent antigen. The evidence is therefore strong that keratinocyte damage in lichen planus is lymphocyte mediated, but little is known of the mechanism by which it occurs. HLA-DR and ICAM-1 expression on keratinocytes is likely to be an important mechanism in lymphocyte accumulation, but again the trigger for their expression remains obscure.

In conclusion, whilst the mechanisms of cell damage in lichen planus can be explained, the true nature of the disease remains elusive. The true status of lichenoid drug symptoms remains to be established, and the mechanism by which malignant transformation occurs also demands further research.

### 8.6 Treatment

There is not at present a wholly effective treatment for lichen planus, especially in its erosive forms such as desquamative gingivitis. Topical steroids and intralesional steroid injections are the principal forms of treatment, but griseofulvin may also be effective in patients with erosive lesions. A proportion of patients with focal lichenoid reactions adjacent to amalgam restorations have been shown on patch testing to be allergic to mercury (LAINE et al. 1992). It has therefore been proposed in patients with such lesions that patch testing should be carried out and, if a positive result is obtained, the amalgam restorations be replaced. Other forms of treatment include mouthwashes and avoidance of irritants.

It seems probable that, once the real nature of lichen planus and its relationship to lichenoid eruptions have been elucidated, rational and effective treatments are likely to follow.

## 9 Conclusion

In spite of the similar clinical presentations of many of the mucocutaneous conditions described here, the molecular basis of their pathogenesis has been elucidated by recent research. It is to be hoped that this will lead to more precise clinical diagnosis and more rational strategies for treatment.

The process of accurate clinical diagnosis demands a thorough history of the duration, location and frequency of lesions, and it needs to be determined whether there are any specific triggers, such as medication or preceding herpes infection. Given the likely genetic susceptibility to a number of these diseases, the existence of a family history of the disease is particularly important. As well as establishing the characteristics of oral mucosal lesions, it is also crucial to ascertain whether other mucosal sites are affected and whether skin lesions are present. Ophthalmic examination is indicated in the case of MMP and similar diseases. Patch testing is indicated in lesions such as localised forms of lichen planus where a hypersensitivity reaction is suspected.

Routine histological examination is mandatory in the diagnosis of most of the diseases discussed in this chapter, taking care to include perilesional mucosa in the biopsy so that the diagnosis is not obscured by secondary inflammatory changes. The diagnosis of pemphigus and pemphigoid requires histopathological examination, and direct and indirect IF are important in these and similar diseases. In diseases such as those of the EB complex, ultrastructural examination is also indicated to determine the site and nature of the lesion.

There may remain some cases where a differential diagnosis cannot be resolved. However, the specific characterisation of the antigens involved in many of these diseases now raises the possibility of accurate diagnosis by immunoprecipitation of circulating antibodies or their detection using enzyme-linked immunosorbent assay (ELISA)-based systems. It is anticipated that within the next decade specific molecular diagnosis of this group of clinically similar mucocutaneous diseases should be possible.

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# New Aspects of Oral Viral Diseases

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## 1 Introduction

The past decade has seen intense and increasing interest in the oral health care consequences of viral infection, particularly the possible relationships between viruses and oral diseases – especially malignant neoplasms, the possible infectivity of saliva and oral secretions and, latterly, the oral consequences of infection with the human immunodeficiency viruses (HIV).

Apart from HIV, most viruses known to cause significant oral disease are DNA viruses, all capable of latency and of being reactivated to cause shedding and disease, not least by loss of immune competence. These infections thus figure large in causing the oral lesions in HIV-infected and other immunocompromised individuals and in being potential sources of infection. Amongst such infective agents, the most important are the herpesviruses and papillomaviruses. These are discussed in this chapter, which is focused on the developing understanding over the decade up to 1995 of the relationship between viruses and oral mucosal lesions (Table 1).

Space here precludes coverage of the more tenuous relations of viruses with oral and salivary gland disease, of salivary carriage of viruses, and of hepatitis, HIV, human T cell leukaemia virus (HTLV)-I, HTLV-II, measles, mumps, rubella and other viruses. Possible virally induced mucosal disorders such as Kawasaki's disease (mucocutaneous lymph node syndrome) and possible relationships between hepatitis viruses and other oral disease, such as that between hepatitis C and Behcet's syndrome, hepatitis C and oral cancer, and hepatitis C and lichen planus, are not discussed here; details can be found elsewhere (SCULLY et al. 1990, 1991; SCULLY and BAGG 1992; SCULLY and SAMARANAYAKE 1992; SCULLY 1993, 1995; PEDERSEN et al. 1993; NAGAO et al. 1995).

There are at least seven, now possibly eight known herpesviruses (Table 2) and, of these, oral lesions have been firmly attributed to at least five, namely herpes simplex viruses (HSV) types 1 and 2, varicella zoster virus (VZV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV). All can cause a primary infection which is generally subclinical, and all remain latent thereafter and can be reactivated,

**Table 1.** Viral infections with oral manifestations

| Virus implicated | Major proven oral manifestations |
|---|---|
| Herpes simplex-1 | Herpetic stomatitis<br>Herpes labialis<br>Recurrent intra-oral ulcers<br>Erythema multiforme |
| Herpes simplex-2 | Herpetic stomatitis<br>Herpes labialis<br>Erythema multiforme |
| Herpes varicella zoster | Pain and ulceration |
| Epstein-Barr virus | Ulceration<br>Lymphomas<br>Palatal petechiae<br>Hairy leukoplakia |
| Cytomegalovirus | Ulceration |
| Human herpesvirus-6 | ? |
| Human herpesvirus-7 | ? |
| Kaposi's sarcoma herpesvirus | ? Kaposi's sarcoma |
| Human papillomaviruses | Papillomas, verruca vulgaris, condyloma acuminatum<br>focal epithelial hyperplasia |
| Molluscum contagiosum | Labial papules |
| Orf | Labial nodule |
| Measles | Koplik's spots |
| Rubella | Palatal petechiae |
| Mumps | Sialadenitis |
| Coxsackie/echo viruses | Ulceration |
| Human immunodeficiency viruses | Fungal infections<br>Viral infections<br>Auto-immune disease (e.g. petechiae)<br>Tumours |

**Table 2.** Human herpesviruses

| Herpesviruses | Abbreviation |
|---|---|
| 1 Herpes simplex type 1 | HSV-1 |
| 2 Herpes simplex type 2 | HSV-2 |
| 3 Varicella zoster virus | VZV |
| 4 Epstein-Barr virus | EBV |
| 5 Cytomegalovirus | CMV or HCMV |
| 6 Human herpesvirus-6 | HHV-6 |
| 7 Human herpesvirus-7 | HHV-7 |
| 8 Kaposi's sarcoma herpesvirus | KSHV |

sometimes with resultant disease. Some herpesviruses are oncogenic. In the immunocompromised host, virus infection or reactivation can cause severe and sometimes even fatal disease.

There are over 70 human papillomaviruses known (HPV), some of which cause oral lesions alone, some also lesions affecting skin or other mucosae (Table 3). Most HPV cause benign lumps such as papillomas or warts, but the possible

**Table 3.** Human papillomavirus-induced cutaneous and mucosal lesions

| HPV type | Clinical features |
|---|---|
| 1a,b,c | Deep plantar warts |
| 2a–e | Common warts (verruca vulgaris)<br>Mosaic plantar warts<br>Oral verrucous carcinoma<br>Oral warts |
| 3a,b | Flat warts (juvenile warts)<br>Warts in EV (non-malignant) |
| 4 | Small palmar and plantar warts<br>Oral warts |
| 5a,b | Warts in EV (malignant)<br>Skin carcinoma (in immunosuppressed patients) |
| 6a–f | Vulvar carcinoma<br>Laryngeal papillomas<br>Genital condyloma<br>Buschke-Lowenstein tumour<br>Cervical intra-epithelial neoplasia<br>Oral papillomas/condylomas |
| 7 | Common warts (Butcher's warts)<br>Oral warts (in immunosuppressed patient) |
| 8 | Warts in EV<br>Squamous cell carcinoma in EV |
| 9 | Warts in EV (non-malignant) |
| 10a,b | Flat warts |
| 11a,b | Oral papillomas/condylomas<br>Laryngeal papillomas<br>Laryngeal carcinomas<br>Cervical intra-epithelial neoplasia<br>Genital warts<br>Penile carcinoma |
| 12 | Warts in EV |
| 13a,b | Focal epithelial hyperplasia (Heck's disease) |
| 14a,b | Warts in EV<br>Squamous cell carcinoma in EV |
| 15 | Warts in EV (non-malignant) |
| 16 | Carcinoma of oesophagus<br>Carcinoma of larynx<br>Oral carcinoma<br>Genital condyloma<br>Cervical intra-epithelial neoplasia<br>Bowenoid papulosis<br>Carcinoma of cervix<br>Carcinoma of penis<br>Carcinoma of anus<br>Lung carcinoma |
| 17a,b | Warts in EV<br>Squamous cell carcinoma in EV |

**Table 3.** *Continued*

| HPV type | Clinical features |
|---|---|
| 18 | Carcinoma of cervix<br>Carcinoma of penis |
| 19 | Warts in EV |
| 20 | Warts in EV<br>Squamous cell carcinoma in EV |
| 21–25 | Warts in EV |
| 26 | Verruca vulgaris (in immunosuppressed patients) |
| 30 | Laryngeal carcinoma<br>Cervical intra-epithelial neoplasia |
| 31 | Carcinoma of cervix |
| 32 | Focal epithelial hyperplasia (Heck's disease)<br>Oral papilloma |
| 33 | Cervical intra-epithelial neoplasia<br>Carcinoma of cervix<br>Tonsillar carcinoma |
| 34 | Non-genital Bowens disease<br>Cervical intra-epithelial neoplasia |
| 35 | Cervical intra-epithelial neoplasia<br>Carcinoma of cervix |
| 36 | Warts in EV<br>Actinic keratosis |
| 37 | Keratoacanthoma |
| 38 | Melanoma |
| 39 | Bowenoid papulosis<br>Cervical intra-epithelial lesion<br>Cervical carcinoma |
| 40 | Laryngeal carcinoma |
| 41 | Vulvar papilloma<br>Cervical intra-epithelial lesion |
| 42 | Flat condylomas (Bowenoid lesions) |
| 43/44 | Low-grade epithelial dysplasias of genital epithelium |
| 45 | Cervical intra-epithelial neoplasia<br>Cervical carcinoma |
| 46/47 | Warts in EV |
| 48 | Squamous cell carcinoma in EV |
| 49/50 | Warts in EV |
| 51/52 | Cervical intra-epithelial neoplasia<br>Carcinoma of cervix |
| 53 | Cervical intra-epithelial neoplasia |
| 54 | Genital condyloma |
| 55 | Bowenoid papulosis |
| 56 | Cervical intra-epithelial neoplasia<br>Carcinoma of cervix |

Table 3. *Continued*

| HPV type | Clinical features |
|---|---|
| 57 | Cervical intra-epithelial neoplasia<br>Nasal papilloma<br>Oral papilloma |
| 58 | Cervical intra-epithelial lesion |
| 59 | Skin warts |
| 60 | Cervical intra-epithelial lesion |
| 61 | Vaginal intra-epithelial lesion |
| 62 | Vaginal intra-epithelial lesion |
| 63 | Skin warts (foot) |
| 64 | Vaginal intra-epithelial lesion |
| 65 | Skin warts (finger) |
| 66 | Carcinoma of cervix |
| 67 | Vulval intra-epithelial lesion |
| 68 | Vulval intra-epithelial lesion |
| 69 | Cervical intra-epithelial lesion |
| 70 | Papilloma of vulva |
| 71 | Vaginal intra-epithelial lesion |

EV, epidermodysplasia verruciformis. After DE VILLIERS 1989 and ZUR HAUSEN and DE VILLIERS 1994.

association of these viruses with oral carcinoma is the focus of intense attention and is therefore discussed in some detail below.

## 2 Herpesviruses and Herpes-like Viruses

### 2.1 Herpes Simplex Viruses

HSV are definitely the causal agents of a range of orofacial lesions, including primary herpetic stomatitis, recurrent herpes labialis and recurrent intra-oral infections. HSV is also implicated in many instances of recurrent erythema multiforme. HSV might play a role in some cranial neuropathies, Behcet's syndrome, and other oral ulcers and oral squamous carcinoma.

There are two subtypes, HSV-1 and HSV-2, sharing considerable homology. Different strains are found in different geographical regions, and some strains vary in, for example, their neuro-invasive qualities (SAKAOKA et al. 1987; BERGSTROM and LYCKE 1990). HSV subtypes and strains can be differentiated by DNA restriction-endonuclease analysis (herpesvirus fingerprinting).

Enclosing the HSV DNA genome is a protein capsid, and the whole is enclosed in a lipid-containing envelope derived from the nuclear membrane of the host cell.

This envelope contains glycoproteins (gp), some of which are responsible for HSV infectivity: for example, gpB and gpC may be responsible for attachment to cell surface receptors.

Recent advances in the understanding of HSV epidemiology, pathogenesis and treatment are reviewed elsewhere (COREY and SPEAR 1986; WILDY 1985; SCULLY 1989, 1995; SCULLY et al. 1991; MILLER and REDDING 1992; SCULLY and SAMARANAYAKE 1992; VESTEY and NORVAL 1992; SYRJANEN 1992).

### 2.1.1 Natural History of Infection

HSV binds by envelope glycoproteins to epithelial cell surface receptors which are heparan sulphate proteoglycans (WUDUNN and SPEAR 1989). The nucleocapsid is then released into the cell cytoplasm. The nucleocapsid releases HSV DNA, which reaches the cell nucleus.

Alpha genes ("immediate early" or IE genes) are then expressed, followed by beta genes ("early" or E genes), which produce regulatory proteins and enzymes needed for DNA replication. The virus thus shuts down most cell protein synthesis while initiating viral gene transcription. Later, the gamma genes ("late" or L genes) that specify HSV structural proteins are expressed (O'HARE and HAYWARD 1985). As each category of gene is expressed, the former is turned off. The HSV genome is then replicated, structural proteins synthesised and nucleocapsids assembled (appearing as nuclear inclusion bodies), and these pass through the nuclear membrane (thereby acquiring the envelope) into the cytoplasm and thence to the cell surface. The cell typically dies as the virus is released to spread to adjacent cells, and intra-epithelial vesiculation results from this cytopathic effect.

HSV also infects the trigeminal nerve, where it remains latent in the ganglion and can be reactivated, sometimes with clinical recurrent infection (STRAUS et al. 1985).

### 2.1.2 Epidemiology of Infection

Neonates are often protected by maternal antibodies to HSV that have crossed the placenta. Oral HSV infection is seen mainly in pre-school children, most commonly in lower socio-economic classes (ADES et al. 1989). By the age of 15 years, 50% of the population has been infected by HSV-1 (CHRISTENSON et al. 1992), and by adult life some 63% have antibody evidence of infection by HSV-1 (BLACKWELDER et al. 1982). Infections are, however, now seen increasingly in older children and adults in developed countries.

HSV-1 is usually responsible for herpetic stomatitis, but HSV-2 may also cause oral lesions (LOWHAGEN et al. 1990; GUINAN et al. 1985; SACKS 1984; STRAND et al. 1986; COREY 1988; LAFFERTY et al. 1987). There may be concurrent oral and genital infections with either HSV-1 or HSV-2 or both, but previous oral HSV-1 infection may protect to some extent against genital infection with HSV-2, probably by means of a common mucosal antibody response (ASHLEY et al. 1994).

### 2.1.3 Transmission

A total of 5%–8% of children and 2%–10% of adults periodically shed infectious HSV in saliva even in the absence of clinical lesions (OVERALL 1984). Shedding is more common in immunocompromised subjects. Infection is contracted from HSV lesions or infected secretions such as saliva (SPRUANCE 1984). Failure to follow cross-infection control procedures in oral health care facilities may lead to spread of infection to staff (ROWE et al. 1982; PERL et al. 1992) or to patients (MANZELLA et al. 1984; PERL et al. 1992).

### 2.1.4 Clinical Features of Oral Primary Infections

Many primary infections with HSV are subclinical or pass unrecognised. Stomatitis and pharyngitis are the most frequent clinical manifestations of primary oral infection (MCMILLAN et al. 1993). The clinical picture is fairly distinct, with mouth ulcers, gingivitis, fever and cervical lymphadenopathy. The saliva contains large quantities of HSV. In children, there are occasionally extra-oral manifestations such as rashes (SCULLY 1985). The illness tends overall to be more severe in adults.

Chronic and progressive oral HSV infection may be seen in immunocompromised individuals (COREY and SPEAR 1986; GRATTAN et al. 1986), including those with HIV infection (JONES et al. 1992; discussed elsewhere), while those with atopic eczema may develop widespread skin and sometimes visceral infection (eczema herpeticum).

### 2.1.5 Immunology

Intact epithelium constitutes the main defence against HSV infection, though humoral and cellular responses directed against cell surface viral glycoproteins are also important. The latter is the most important mechanism for recovery and subsequent control of HSV infection (WILDY and GELL 1985; KOHL 1985; ROUSE 1985).

Antibodies also mediate viral neutralisation and antibody-dependent cellular cytotoxicity (ADCC). Responses to IE, E and L proteins (structural and non-structural proteins), especially to the IE proteins ICP4 and ICPO, are seen early in primary infection.

### 2.1.6 Latency

Latent infections are persistent infections where the viral genome is present but the gene expression is limited and no infectious virus is produced. The mechanisms are fully reviewed elsewhere (ROIZMAN and SEARS 1987; BANKS and ROUSE 1992). HSV resides in neuronal cells such as the trigeminal ganglion. HSV DNA can also be found in some clinically normal oral epithelium (COX et al. 1993).

Latent virus expresses early replicative functions, but there is a block in HSV transcription and neither late viral antigens nor free virus can be detected (STANBERRY 1986). Furthermore, the neurones in which HSV is latent express no major histocompatitbility antigens (MHC) antigens and thus, even were viral antigens to be produced, they could not be presented to T lymphocytes. HSV glycoprotein gPG can bind complement component C3b and thereby impede the alternative complement pathway, offering another mechanism of evading immune attack.

Finally, though the mechanisms are unclear, it is clear that herpesviruses can be immunosuppressive and that HSV (probably via a viral glycoprotein) can impair a range of immune functions, including functions of natural killer (NK) cells, neutrophils and macrophages (BANKS and ROUSE 1992). Herpesvirus infections can thus exacerbate the immune defect in HIV and other infections.

### 2.1.7 Reactivation

Trigger factors appear to operate either via derepression of the latent viral genes or deregulation of immune surveillance mechanisms. Genetic factors must also be at play, as only about one third of non-immunocompromised patients have clinical recurrences.

Triggers for HSV reactivation include trauma (BARKVOLL and ATTRAMADOL 1987), including that of dental extraction, facial fractures and of decompression of the trigeminal nerve (KAMEYAMA et al. 1988, 1989), ultraviolet light (SPRUANCE 1985), fever or immunosuppression (HILL 1985; GREENBERG et al. 1987) and radiotherapy (REDDING 1990). Other factors that are often stated to be associated with recurrent herpetic infection include menstruation and other hormonal changes, but there appear to be no studies that confirm this.

Although it is often suggested that stress can also lead to recurrent infections, few studies have been done, and their results are inconsistent, though it has been reported that levels of serum antibody to HSV-1 are elevated in medical students during and just before examinations (GLASER et al. 1985, 1987), in separated and divorced men (KIECOLT-GLASER et al. 1988) and in individuals living near the Three Mile Island nuclear plant in the United States (MCKINNON et al. 1989). Though the relationship between stress, immune function and various infectious diseases is an area of much current interest (COHEN and WILLIAMSON 1991), the association between recurrent infections by HSV-1 and psychological stress remains to be proved.

Associations of recurrences of HSV infection with HLA-A1, HLA-B5 and HLA-DR1 and a decreased frequency of HLA-B35 have been shown, but other reported HLA associations are equivocal (LEGENDRE et al. 1982; GALLINA et al. 1985; JABBAR et al. 1991).

Reactivation of HSV is more common than clinical recurrence (HARBOUR et al. 1983) and still occurs in the presence of high titres of neutralising specific antibody. There is some evidence that prostaglandin $E_2$ may, by depressing ADCC and production of interleukin-2, reactivate HSV (BLYTH and HILL 1984).

Immunoglobulin G (IgG) antibodies to internal capsid protein and a range of structural proteins (gB, gD, VP19, VP20 and VP23), but not IE proteins,

are found in recurrences (WILDY 1986; KUHN et al. 1987). An increased titre of antibody to gD is associated with reduced recurrences of HSV, while deficient antibody to VP66 is associated with increased disease severity (BERNSTEIN et al. 1987).

The protective role of cellular immune responses is emphasised by the frequent and severe episodes of HSV infection seen in patients with defective cellular immunity. Multiple immunocyte populations, including various T cell subsets (especially cytotoxic T cells), macrophages, NK cells and natural cytotoxic cells, are involved (ROUSE 1985; RAGER-ZISMAN et al. 1987).

Recurrences are presumably because of transient immunodepression with reduced natural cytotoxic cell activity, altered neutrophil motility and lymphoproliferative responses and reduced levels of some lymphokines, such as leucocyte migration inhibition factors and immune interferon (GREEN et al. 1985).

Clinical lesions in reactivated HSV infection are usually at the mucocutaneous junction on the lips (herpes labialis). Why, in otherwise healthy patients, recurrent HSV infections are usually labial and not intra-oral is unclear, but may relate to oral protective factors such as salivary IgA or lysozyme.

If intra-oral lesions arise in normal subjects, they are typically on the palate or gingiva. In immunocompromised subjects, intra-oral lesions are usually progressive linear ulcers, often on the tongue (GROSSMAN et al. 1993), and may be related to reduced ADCC activity.

There is increasing evidence of intra-oral recurrences, notably as chronic ulcers in the mouth, especially in leukaemic or other immunocompromised persons (GREENBERG et al. 1987a,b; BARRETT 1986, 1987; COHEN and GREENBERG 1985; MONTGOMERY et al. 1986; BERGMANN et al. 1990). Recurrence rates of oral HSV infections are unclear and figures vary from 16% to 45%.

### 2.1.8 Diagnosis of Infections

Many primary infections by HSV-1 can be diagnosed by clinical examination, and no laboratory diagnostic tests are necessary. However, in cases of immunocompromised individuals having lesions with an atypical appearance, laboratory testing can be valuable.

The most useful specimen consists of fluid from an intact vesicle or lesion, since this has a very high concentration of virus particles. The fluid can be adsorbed onto a cotton swab, provided that the swab is kept in viral transport medium and arrives at the laboratory within a few hours (GONIK et al. 1991).

The standard technique for HSV diagnosis of inoculation of a specimen onto susceptible cells examined daily for a cytopathic effect or the use of specific antisera can take several days, but there are now more rapid and more acceptable methods available.

HSV on oral swabs (or that that has started to grow in cell culture) can be detected by immunological methods to demonstrate viral antigens (FOX et al. 1987; LIPSON et al. 1991), giving a diagnosis within 5h of the specimen arriving at the laboratory, with a sensitivity of 88%–99% and a specificity of 90%–100% (VERANO and MICHALSKI 1990; MACPHAIL et al. 1995). However, these rapid methods

require up to 100 times more virus in the specimen that do conventional methods and, with specimens from asymptomatic persons, the sensitivity may drop to below 60% (VERANO and MICHALSKI 1990).

Simple diagnostic kits that may be used directly in the dental office are now available. Examples are the Kodak SureCell assay (FERRIS and FISHER 1992) and SYVA MicroTrak (MACPHAIL et al. 1995). These give a result within 15 min, but suffer from the same disadvantages of sensitivity and specificity as the laboratory-based rapid methods, and the shelf-life of the kits is usually short (only a few months). For vesicular lesions due to HSV, the sensitivity has been reported to be as high as 100%, but when infections are at a non-vesicular stage the sensitivity drops to as low as 76% (DORIAN et al. 1990) or even lower (ZIMMERMAN et al. 1991). Thus a negative result should be confirmed by a standard laboratory culture.

Other rapid laboratory methods use DNA hydridisation, but reported sensitivities are as low as 25% (SEAL et al. 1991). The polymerase chain reaction (PCR), however, is proving a reliable and sensitive rapid method for detecting HSV on smears, though it is not diagnostically superior to viral culture (NAHASS et al. 1992).

## 2.1.9 Management

*2.1.9.1 Primary Stomatitis.* HSV-induced oral lesions are managed mainly with supportive treatment, particularly maintenance of fluid intake, antipyretics, analgesics and topical antiseptics to prevent bacterial superinfection. Antivirals are

**Table 4.** Indications for acyclovir therapy

| Type of infection | Route and dosage[a] |
|---|---|
| Mucocutaneous HSV in an immunocompromised patient | 200–400 mg orally five times/day or 5 mg/kg intravenously every 8 h for 7–10 days[b]<br>5% cream topically every 6 h for 7 days |
| HSV encephalitis | 10 mg/kg intravenously every 8 h for 10–14 days[c] |
| Neonatal HSV | 10 mg/kg intravenously every 8 h for 10–14 days[c] |
| Varicella in normal host | 20 mg/kg orally four times/day for 5 days (maximal dose 800 mg/day) |
| Varicella in an immunocompromised patient | 10 mg/kg intravenously every 8 h for 7–10 days[c] |
| Herpes zoster in a normal host | 800 mg orally five times/day for 7 days |
| Herpes zoster in an immunocompromised patient | 10 mg/kg intravenously every 8 h for 7–10 days |

Modified from WHITLEY and GNANN (1992).
HSV, herpes simplex virus.
[a] The doses are for adults with normal renal function unless otherwise stated.
[b] A dose of 250 mg/m$^2$ body surface area should be given to children under 12 years of age.
[c] A dose of 500 mg/m$^2$ body surface area should be given to children under 12 years of age.

**Table 5.** Antiviral therapy of oral herpes simplex virus (HSV) infection

| Disease | Otherwise healthy patient | Immunocompromised patient |
|---|---|---|
| Primary herpetic gingivostomatitis | Consider oral acyclovir 100–200 mg five times a day[a] | Acyclovir 250 mg/m$^2$ intravenously every 8 h or 400 mg orally five times a day |
| Recurrent herpetic lesions, e.g. herpes labialis | Acyclovir 5% cream | Consider systemic acyclovir as above, depending on risk to patient of infection |

[a] See text for details.

indicated predominantly for immunocompromised patients or where there are frequent severe recurrences or complications (Tables 4, 5). Acylovir, active against HSV thymidine kinase, is still the antiviral of most proven efficacy and safety.

Non-immunocompromised patients with primary HSV stomatitis generally present for treatment with lesions in a late stage of development, and the general view is that acyclovir is then unlikely to be of especial value, *may* elicit resistant HSV, and is therefore not indicated (Table 4).

*2.1.9.2 Recurrences.* Acyclovir 5% cream may shorten or abort recurrences of herpes labialis (GIBSON et al. 1986), but must be applied in the early prodromal phase to have effect in the immunocompetent patient. Use later in the disease is unlikely to be beneficial and is possibly contraindicated, bearing in mind the theoretical possibility of producing viral resistance (Table 5).

Oral acyclovir has been shown to produce marginal improvement in herpes labialis and suppresses recurrences (RABORN et al. 1987). Long-term suppressive therapy using 200 mg acyclovir orally four times a day may significantly suppress recurrences; though associated with no important side effect (RABORN et al. 1988), it should be avoided in pregnancy.

Acyclovir also appears to be clinically beneficial in treatment of herpetic whitlows (LASKIN 1985), eczema herpeticum (TAIEB et al. 1985), herpetic encephalitis (WHITLEY et al. 1986) and post-herpetic erythema multiforme (LYNN et al. 1987).

Because reactivation of HSV is a major cause of morbidity in immunocompromised patients, producing prolonged pain and occasionally mucocutaneous or visceral dissemination, systemic acyclovir can then be of substantial value (GLUCKMAN et al. 1983). Viral shedding, pain and duration of lesions are substantially reduced using acyclovir, either intravenously at a dose of 250 mg/m$^2$ body surface area every 8 h or orally 400 mg five times a day (SHEPP et al. 1985). However, treatment of established lesions in immunocompromised patients is less satisfactory than prophylaxis, and it has been suggested that prophylaxis might actually minimise, rather than increase, the risk of acyclovir resistance. Acyclovir has been advocated for prophylaxis in immunocompromised adults using an oral dose of 200 mg three to four times daily (MINDEL 1991) or acyclovir topically.

Acyclovir resistance is now becoming a clinical problem, however (EPSTEIN and SCULLY 1991). CRUMPACKER (1988) found that 7% of HSV isolates were

acyclovir resistant, mostly those from immunocompromised hosts. Significant clinical infections with acyclovir-resistant HSV are now being reported with increasing frequency (Westheim et al. 1987; Schinazi et al. 1986), particularly in patients with leukaemia, after tissue and organ transplants and in those with HIV disease (Schinazi et al. 1986; Norris et al. 1987; Erlich et al. 1989; Youle et al. 1988; MacPhail et al. 1989).

Most acyclovir-resistant HSV isolates are thymidine kinase deficient (Crumpacker 1988) but are, fortunately, still sensitive to foscarnet (trisodium phosphonoformate hexahydrate) (Youle et al. 1988; MacPhail et al. 1989). Foscarnet inhibits HSV-specific DNA polymerase and has low toxicity to mammalian cells (Ringden et al. 1986).

### 2.1.10 Erythema Multiforme

Erythema multiforme is a recurrent condition characterised by a rash, oral and labial erosions and conjunctivitis in various combinations, which may be precipitated by a range of factors, particularly drugs and micro-organisms. Most cases have thus far been regarded as idiopathic, since no precipitant was known, though the association of some erythema multiforme with HSV has always been well recognised (Grimwood et al. 1983; Lozada-Nur and Shillitoe 1985; Huff and Weston 1989). However, a history of HSV infection often precedes recurrent erythema multiforme (Leigh et al. 1985); HSV antigens have now been found in circulating immune complexes (Kazmierowski et al. 1982; Orton et al. 1984) and can sometimes be demonstrated in cutaneous (Orton et al. 1984) and oral lesions (Malmstrom et al. 1990), even though HSV cannot be regularly cultured or identified electron-microscopically (Leigh et al. 1985; Huff and Weston 1989). HSV DNA has now been demonstrated in lesional tissue by in situ hybridization (Brice et al. 1989), and PCR has now shown HSV DNA to be present in up to 80% of cases (Aslanzadeh et al. 1992; Brice et al. 1989; Darragh et al. 1991; Weston et al. 1992; Miura et al. 1992). It is clear, therefore, that much of what has been regarded as idiopathic recurrent erythema multiforme is, in fact, HSV related.

A combination of immune reactions, particularly immune complex formation to HSV, probably cause this type of erythema multiforme rather than any epidermotropic viral effect of HSV (Leading Article 1989). Individuals who have the genetic background of HLA-B15 (Duvic et al. 1983) and HLA DQw3 (Kampgen et al. 1988) appear to be predisposed to HSV-induced erythema multiforme; there are as yet no defined immune response differences between those who do and those who do not develop erythema multiforme (Brice et al. 1993).

Antivirals such as acyclovir can control recurrences of HSV-related erythema multiforme (Molin 1987; Green et al. 1985; Lemak et al. 1986), though, interestingly, HSV DNA remains in the skin despite continuous acyclovir therapy (Miura et al. 1992). In severe cases, corticosteroids may also be indicated (Detjen et al. 1992), or levamisole may be of value (Lozada-Nur et al. 1992).

### 2.1.11 Lichen Planus

HSV DNA may sometimes be found in lichen planus, but HSV is not necessarily causal (Cox et al. 1993).

### 2.1.12 Keratoses

Again, HSV DNA may be found in keratoses (Cox et al. 1993), but the association may not be causal.

### 2.1.13 Oral Carcinoma

The current evidence highlights the role of papillomaviruses rather than HSV in oral carcinoma, as discussed later. HSV-1 is nevertheless clearly oncogenic. HSV is capable of transforming cells *in vitro*, provided cytolysis is inhibited (DUFF and RAPP 1971; RAPP 1981), by factors such as ultraviolet light (RAPP 1981) and certain chemicals (HIRSCH et al. 1984). In some *in vitro* systems such as SV40-transformed hamster embryo cells, HSV is more effective than some chemical carcinogens in amplifying SV40 DNA sequences (SCHLEHOFER et al. 1983; MATZ et al. 1984), acting via HSV-encoded DNA polymerase (MATZ et al. 1984, 1985). Several reports indicate that HSV acts synergistically with chemical carcinogens in causing oncogenic transformation (JOHNSON 1982; KOCERA et al. 1983; PARK et al. 1991), and it is now clear that HSV is synergistic with tobacco-specific nitrosamines in cell transformation.

*In vitro* HSV induces chromosomal aberration, mutations and gene amplification, and in the hamster cheek pouch model of dimethylbenzanthracene-induced carcinogenesis it enhances *erbB1* oncogene amplification and overexpression (OH et al. 1989), a feature that coincides with the appearance of malignancy. HSV also binds to the receptor for basic fibroblast growth factor, and this interaction might conceivably activate *myc* and other oncogenes.

Animal studies suggest that HSV may be a co-carcinogen with tobacco or other chemicals (HIRSCH et al. 1984; PARK et al. 1986; LARSSON et al. 1989; OH et al. 1989) and that immunisation against HSV prevents the co-carcinogenic activity of HSV with dimethylbenzanthracene (PARK et al. 1990). Substantial evidence suggests, therefore, that HSV might under particular circumstances be oncogenic.

Studies of the association of HSV with oral carcinoma have shown interesting results. A number of studies have shown changes in levels of serum antibodies to HSV patients with oral carcinoma (LARSSON et al. 1991; VASUDEVAN et al. 1991; SHILLITOE et al. 1982, 1983; KUMARI et al. 1985). For example, serum IgA antibodies to HSV-1-induced antigens may be increased in tobacco smokers, whether they have oral carcinoma or not, but the increases in smokers without tumours are less than in those with carcinomas.

There is a higher reactivity to the HSV IE protein ICP4 in patients with oral cancer, suggesting a different course of an earlier herpetic infection, with a prolonged exposure to IE proteins of HSV as a consequences of smoking

(LARSSON et al. 1991). Smoking may act, at least in part, by suppressing NK cell activity, which is involved in control of HSV. Indeed, there are close relationships between NK cell activity and antibody production to HSV in patients with carcinoma of the head and neck (SCHANTZ et al. 1986). Systemic factors often associated with oral carcinoma, such as alcohol and liver disease, might also impair NK cell activity.

Examination of oral carcinoma tissues for HSV viral "footprints" has given interesting, but equivocal results, though failure to demonstrate HSV products does not, of course, exclude a "hit and run" mechanism. HSV antigens have been shown in carcinomas in some, but not all studies. Our demonstration, using *in situ* hybridisation, of RNA complementary to HSV DNA in biopsy specimens from oral carcinoma but not from autologous, clinically normal oral mucosa suggested an association of HSV with oral carcinoma (SCULLY et al. 1982; EGLIN et al. 1983), and others have since demonstrated HSV-1 DNA in oral carcinoma tissue (VASUDEVAN et al. 1991). However, this is not *proof*, since hybridisation could be revealing segments of normal host nucleic acid with homology to part of the HSV genome.

Therefore, the evidence for an association of oral carcinoma with HSV, though stronger than for other herpesviruses or adenoviruses, is not unequivocal. However, carcinogenesis is not a single-step procedure with a single aetiology, and it has been suggested that HSV may act synergistically with HPV in carcinogenesis. With regards to cervical carcinoma, epidemiological evidence indicates that this may be possible and, in experimental situations, it has been demonstrated that keratinocytes immortalised by HPV-16 DNA are tumourigenic in nude mice following transfection with HSV DNA (IWASAKA et al. 1988; DIPAOLO et al. 1990).

Further studies are needed to investigate the role of HSV in oral carcinogenesis. The possible association of HSV with carcinoma is discussed fully elsewhere (PARK et al. 1992; SCULLY 1983, 1992; SCULLY et al. 1991; SCULLY and SAMARANAYAKE 1992; SHILLITOE 1991; LARSSEN et al. 1991).

### 2.1.14 Aphthae

The controversial possible association of HSV with aphthae and Behcet's syndrome is discussed elsewhere (SCULLY et al. 1991; SCULLY 1993; PEDERSEN et al. 1993).

## 2.2 Herpes Varicella Zoster Virus

VZV, sometimes termed herpesvirus-3, can cause chickenpox (varicella) or shingles (zoster) (STRAUS et al. 1988; HYMAN 1987; SCULLY and SAMARANAYAKE 1992; SCULLY, 1995).

There appears to be only one type of VZV. The virus contains five glycoproteins, of which the main ones are gpI, gpII and gpIII (DAVISON et al. 1986),

antibodies against these glycoproteins having neutralizing activity (GROSE and LITWIN 1988; BRUNELL et al. 1987).

### 2.2.1 Varicella (Chickenpox)

*2.2.1.1 Immunopathogenesis.* VZV infects mainly via the respiratory tract and, after a viraemia, may produce mucocutaneous lesions. Most infections are subclinical, but chickenpox is the clinical manifestation of primary infection with VZV. It is seen predominantly in children, usually in late winter and spring. There appears to be an increasing incidence of chickenpox both in adult immigrants from the developing world and in immunocompromised patients.

The oropharynx is a site for early replication of VZV. The virus then spreads to sensory nerve ganglia, where it becomes latent. Immunity to VZV is mainly cellular. Cytotoxic T cells and ADCC mechanisms appear to be protective, as is interferon, and, where these mechanisms are impaired, there may be severe disease and recurrence (SCULLY, 1995; SCULLY and SAMARANAYAKE 1992).

Infection with VZV usually confers life-long protection except in some immunocompromised patients.

*2.2.1.2 Clinical Features.* The incubation period is 14–21 days. Many primary infections are subclinical or pass unnoticed, but chickenpox may present with malaise, anorexia, irritability and fever followed by vesicles and mouth ulcers mainly on the palate and tongue. There is a rash affecting the scalp and then the face, neck and trunk. Lesions are seen at all stages in development, from itchy, red macules to papules (1–4 mm in diameter), vesicles, pustules and scabs (i.e. the rash is centripetal, and it crops).

*2.2.1.3 Diagnosis.* Diagnosis of chickenpox is clinical, and only enterovirus infections and a few other viral infections need to be excluded. A Tzanck smear from a lesion, culture or electron microscopy can be useful where the diagnosis is in doubt, but PCR detection of VZV DNA is superior (NAHASS et al. 1992).

*2.2.1.4 Complications.* Most patients recover spontaneously in 2–3 weeks, but a few, especially pregnant women, develop complications such as pneumonia (PREBLUD, 1986; PREBLUD et al. 1984). Varicella during pregnancy also poses a risk to the fetus, which may be born with cicatricial scarring and limb deformities and may subsequently also suffer zoster as a child.

Immunocompromised patients can develop widespread and severe disease. Such patients are covered with poxes which also affect oral and other mucosae, liver and spleen.

*Reye's syndrome*, characterised by central nervous system (CNS) symptoms, cerebral oedema and fatty degeneration of the liver, may follow varicella if there is salicylate use: VZV appears to be a factor in 16%–28% of cases.

*2.2.1.5 Management.* Varicella is benign in otherwise healthy persons, and only supportive management is required. However, antivirals such as acyclovir may be

indicated if the patient is immunocompromised. Passive immunisation with varicella zoster immunoglobulin (VZIg) can also modify or prevent varicella in these groups, and a safe and effective varicella vaccine is also now available (GERSHON et al. 1990; ISSACS and MENSER 1990).

### 2.2.2 Zoster (Shingles)

During chickenpox, VZV ascends to the dorsal root ganglion cells, where it becomes latent. When reactivated, VZV is transported via sensory nerve axons to the skin and/or mucosa and may produce zoster.

*2.2.2.1 Epidemiology.* Zoster affects up to 20% of all individuals at some stage in life, but is most common in the elderly and in the immunocompromised. A total of 75% of those affected are more than 45 years old, and some 50% of immunocompromised persons suffer at least one attack of zoster. Occasionally, there are recurrent attacks of zoster in healthy persons, but this is far less common than in immunocompromised persons (SCULLY, 1995).

*2.2.2.2 Clinical Features and Diagnosis.* Most zoster affects elderly patients, usually in the thoracic (56%) or head and neck (13%) regions. In the head and neck region, ophthalmic zoster is about 20 times more common than zoster in other trigeminal divisions, and it threatens the eye.

The rash of zoster resembles that of chickenpox in its development, but it is restricted to a dermatome, i.e. the area of skin (and mucosa) supplied by a sensory nerve. The rash is typically unilateral and in a band-like distribution, hence the terms zoster (Greek) or shingles (Latin), meaning "belt". Macules progress through papules and small vesicles to produce pustules by 3–4 days. If the maxillary or mandibular divisions of the trigeminal nerve are involved, mouth ulceration is usually seen (MILLAR and TROULIS, 1994).

Severe pain often precedes, accompanies and follows the rash of herpes zoster, sometimes persisting for months or years (post-herpetic neuralgia). The pain may simulate toothache. Some 70% of those with zoster, mostly the elderly, have pain persisting after the rash heals, usually for about 1 month, which is the definition of post-herpetic neuralgia. Nearly 30% have neuralgia persisting for up to 6 months, and some longer. Post-herpetic neuralgia may be aggravated by temperature changes and tends to be worse at night, but usually slowly improves and resolves over 1 year or so. Only 2%–5% have pain lasting 1 year or more (WOOD 1991).

The nature of post-herpetic neuralgia varies between patients, but two main types have now been distinguished. The first is a constant pain, and the other a paroxysmal pain, stimulating idiopathic trigeminal neuralgia. Either may be triggered by touching the area.

*2.2.2.3 Variants and Complications.* Zoster is occasionally bilateral (HILL and LAMEY, 1986) or may be generalised (this is usually in the immunocompromised). Very occasionally, oral lesions alone are seen in mandibular or maxillary zoster.

Zoster occasionally occurs without a rash (zoster sine herpete) (BARRETT et al. 1993). Necrosis of jaw bone and, if zoster affects a child, dental hypoplasia and retarded tooth eruption may be seen (SMITH et al. 1984).

*2.2.2.4 Herpes Zoster in the Immunocompromised Patient.* Zoster is common in immunocompromised patients, especially in those with Hodgkin's lymphoma, particularly where there has been radio- or chemotherapy, and in HIV disease. In patients with HIV infectio, zoster can be severe and may be predictive of the development of acquired immunodeficiency syndrome (AIDS) (MELBYE et al. 1987).

Morbidity is high in immunocompromised patients, with persistent zoster lesions, significant tissue destruction, scarring, viral dissemination and more severe post-herpetic neuralgia. The rates of post-herpetic neuralgia in immunocompromised patients range from 18% to 45%, about the same as rates in immunocompetent patients (WOOD, 1991). Chronic zoster and persistent CNS infection with progressive encephalopathy may ocasionally develop (MANDAL 1987; RYDER et al. 1986).

*2.2.2.5 Diagnosis of Oro-facial Zoster.* Diagnosis of zoster is usually clinical and made on the basis of the severe unilateral pain, the unilateral rash restricted to a dermatome, and unilateral mouth ulcers. A Tzanck smear, culture, electron microscopy or serology are very occasionally required for diagnosis (SZAKI et al. 1990; TOVI et al. 1985).

Occasionally, cutaneous recurrent HSV lesions or enterovirus lesions occur in one dermatome and simulate zoster. Hand, food and mouth disease, measles and Rickettsial pox very rarely need to be considered in the differential diagnosis.

*2.2.2.6 Management.* An underlying immune defect should be excluded, but zoster is more commonly simply a consequence of old age.

Treatment is mainly supportive but, in ophthalmic zoster, it is important to seek an early specialist opinion because of the danger to sight. Antivirals such as acyclovir may also be needed, particularly in immunocompromised patients. Oral acyclovir given not later than 72 h after rash onset in a dose of 800 mg five times daily for 7–10 days hastens resolution of lesions and reduces both the acute pain and the incidence and duration of post-herpetic neuralgia (CROOKS et al. 1991). Zoster-immune globulin may also help to control lesions in immunocompromised patients.

Analgesics are indicated to control the pain in zoster, but post-herpetic neuralgia may prove refractory to even potent analgesics. Corticosteroids given in the acute phase may be of some value in preventing the development of pain. Amantadine given in acute zoster or levodopa may be of value in controlling herpetic neuralgia, but tricyclics or an anticonvulsant such as sodium valproate or carbamazepine is usually preferred (LEADING ARTICLE 1990; LOESER 1986). Topical capsaicin, an antagonist of substance P, is of unproven value, but shows promise. EMLA cream may be of some analgesic value (STOW et al. 1989).

Transcutaneous nerve stimulation or ethyl chloride sprays may relieve the pain. Very ocasionally, neurosurgery is indicated; one procedure (dorsal root entry zone coagulation, the Nashold procedure) may produce at least short-term benefit.

*2.2.2.7 Aphthae.* There may be a slight rise in VZV antibody titres in patients with aphthae (PEDERSEN and HORNSLETH 1993). The speculative association of VZV with aphthae is discussed elsewhere (PEDERSEN et al. 1993; PEDERSEN 1989).

*2.2.2.8 Oral Carcinoma.* There is, at least on serological evidence, no association between VZV and oral carcinoma (LARSSON et al. 1991).

## 2.3 Epstein-Barr Virus

EBV is a herpesvirus that infects and replicates in oral and oropharyngeal epithelium and infects, activates and can immortalize B lymphocytes (SCULLY and SAMARANAYAKE 1992; SCULLY 1995).

In developing countries, most children have been infected by EBV, usually asymptomatically, by the age of 18 months. In developed countries, there are two peaks of prevalence of EBV infection, one in pre-school children and the other in teenagers and adolescents (EVANS 1982).

The virus causes the clinical syndrome of infectious mononucleosis, mainly in adolescents of higher socio-economic groups, but it is implicated in a range of other disorders.

### 2.3.1 Virology

EBV consists mainly of a nucleocapsid and an envelope with external glycoprotein spikes containing mainly (gp350/220). EBV genes code nuclear antigens (EBNA) and latent membrane proteins (LMP), as well as others.

At least two EBV types have been identified on the basis of their *Bam*H1 WYH gene, which encodes EBNA-2A (see below). These types, known as EBV-A (or EBV-1) and EBV-B (or EBV-2), are closely related and differ mainly in genes related to the cycle of latent infection. The types are distinguished mainly by the variant of EBNA-2 that they express: EBV-A expresses EBNA-2A, and EBV-B expresses EBNA-2B. They also differ in EBNA-3A, EBNA-3B and EBNA-3C (ROWE et al. 1989; SAMPLE et al. 1990) and in LMP-1 (WALLING et al. 1994; MILLER et al. 1994). Both EBV types are seen worldwide, but EBV-A is the main type in the West, and EBV-B the main type in Africa (SIXBEY et al. 1989).

EBV-A is more prevalent in higher socio-economic classes (YAO et al. 1991). However, immunodeficient individuals, especially those with HIV disease, show more infection with EBV-B or dual infections (SIXBEY et al. 1989; SCULLEY et al. 1990).

### 2.3.2 Immunopathogenesis of Infection

EBV binds to receptors mainly on epithelial cells and B lymphocytes. Lingual epithelium has been shown to have receptors for EBV (CORSO et al. 1989; TALACKO et al. 1991), as have cervical, salivary and lacrimal epithelia. The EBV receptors are identical to receptors for the complement component C3d and are sometimes known as C3dR, CR2 or CD21. The EBV receptor binds to the EBV gp350/220. EBV infects mainly the basal and intermediate layers of epithelium, where it remains latent and, as the epithelial cells differentiate and move towards the luminal surface, the receptor is lost. This whole area is thoroughly reviewed elsewhere (WOLF et al. 1993).

When EBV infects B lymphocytes, some B cells produce EBV early antigen (EA) and viral capsid antigen (VCA) and undergo lysis, but other B cells carry EBV as a latent infection for the rest of the cell's life and express nuclear antigens. Eight genes are expressed: six EBNA and two LMP. The functions of only some antigens are known, but it is clear that EBNA-2 and LMP-1 induce cell proliferation, probably either by stimulating oncogenes or inactivating tumour suppressor genes. EBNA-2 appears to be involved in cell transformation, and LMP interacts with vimentin of the cytoskeleton, acting as an oncogene. EBV can also up-regulate the oncogene *bcl-2* (HENDERSON et al. 1991) and can degrade the tumour suppressor gene product *p53* (FARRELL et al. 1991). *Bcl-2* up-regulation stops programmed cell death (apoptosis) and activates CD23 (see below) and the adhesion molecules lymphocyte function-associated antigen (LFA)-1, intercellular adhesion molecule (ICAM)-1 and LFA-3. LMP can induce phenotypic changes in epithelial cells. EBNA-2, -3 and -6 and LMP are target molecules for T lymphocyte killing.

EBV-infected B cells move into the $G_1$ phase of the cell cycle and begin to express a surface receptor termed CD23 (blast-2), which is a receptor for B cell growth factors and can stimulate the growth of EBV-transformed cells. EBV infection also induces new antigens such as CD30, against which T lymphocytes and NK cells can respond.

Membrane antigens (MA) are expressed only later in EBV infection; one such antigen is detectable only by a T lymphocyte response and is thus termed LYDMA (lymphocyte-detectable membrane antigen). Another, termed gp340, is a principal target for neutralising antibodies. As discussed above, adhesion molecules (LFA-1, ICAM-1 and LFA-3) are also expressed.

EBV infection thus gives rise to two main consequences. Firstly, activated T cells, mainly suppressor (Ts) and cytotoxic (Tc) cells appear in the peripheral blood as atypical mononuclear cells (Downey cells), hence "mononucleosis". Secondly, B lymphocytes, polyclonally activated by EBV, can produce multiple antibodies, including some auto-antibodies and also antibodies reacting to other species (heterophile antibodies). The latter can react with sheep (or cow or horse) erythrocytes, and this forms the basis of the heterophile antibody test (Paul-Bunnell-Davidsohn test).

Immune defences, predominantly T cells, NK cells and other cytotoxic cells, and interferon lyse B cells infected with EBV. Then, some 2 weeks after infection, suppressor T cells appear, which suppress heterophile and other antibody produc-

tion by B cells and suppress the T cell lymphoproliferative responses, thus causing a mild, transient immune defect.

### 2.3.3 Clinical Features of Primary Infection

The incubation period is 30–49 days (mainly 15–25 days). In young children, EBV infection is often asymptomatic or may produce pharyngitis or tonsillitis. In adolescents or adults, EBV typically produces a glandular fever syndrome (infectious mononucleosis) in about 50% of those infected. Sore throat, fever, lymph node enlargement and profound malaise are the main features.

The saliva contains EBV. Oral lesions are seen in about 32% of patients and are the first sign in about half of these. Cervical lymph node enlargement, pharyngitis, creamy white confluent tonsillar exudate, faucial oedema, palatal petechiae, pericoronitis and occasional oral ulceration or acute ulcerative gingivitis are seen. Candidosis may appear if there is significant immune suppression consequent upon the EBV infection.

### 2.3.4 Diagnosis

Investigations may well be indicated, since similar glandular fever-like syndromes can be caused by HIV, human (HCMV), toxoplasmosis, and human herpesvirus-6. Diphtheria may enter into the differential diagnosis of the faucial exudate.

A blood film in infectious mononucleosis shows mononucleosis and atypical lymphocytes (Downey cells); these are large cells with pale-blue vacuolated cytoplasm and an elongated or dented nucleus with coarse chromatin. However, Downey cells, which have T suppressor–cytotoxic activity, are not specific to infectious mononucleosis.

Serodiagnosis is more helpful. Most patients have heterophile antibodies, which are IgM antibodies that appear early in the incubation period, persist for up to 1 year, are detectable by the Paul-Bunnell or Monospot tests and are positive only in infectious mononucleosis. IgG and IgM antibodies to VCA with no antibodies to EBNA is the typical serological pattern.

Throat swabs may be required to exclude diphtheria.

### 2.3.5 Management

Supportive care and rest are indicated. Acyclovir is of little overall benefit in management of infectious mononucleosis. If there is pharyngeal oedema, systemic corticosteroids may be indicated.

### 2.3.6 Complications of Infection

Most patients recover uneventfully, but post-viral fatigue is common. Occasionally, pharyngeal oedema can threaten the airway, and a range of auto-immune

and other complications can arise. Neurological complications, splenic rupture or serious liver disease and Reye's syndrome are rare.

Lymphoproliferative disorders may occur, especially in immunocompromised patients, in HIV disease and in the rare X-linked Duncan's lymphoproliferative syndrome.

*2.3.6.1 Chronic Infection.* EBV may cause unusual or protracted chronic illness with vague malaise, fever, weight loss, hepatosplenomegaly, lymphadenopathy and other features (Leading Article 1985; Beauparlant et al. 1994). However, it is unclear whether EBV is responsible for the chronic fatigue syndrome (CFS), since features of this are very varied and often non-specific.

It is likely that CFS represents a spectrum of illness with various aetiologies such as enteroviruses or possibly human herpesvirus-6.

### 2.3.7 Latency and Reactivation

EBV remains latent mainly in salivary glands. It can be latent in apparently healthy oral mucosa (Loning et al. 1987; Gross et al. 1988; Snijders et al. 1990; Madinier et al. 1992), and in the West this is mainly either EBV-A (50%) or EBV-B (41%), though a minority (9%) have both types (Sixbey et al. 1989). EBV also remains latent in B cells in some long-term carriers and appears to avoid immune detection (Yao et al. 1989). EBV-infected B cells may occasionally transform to lymphoma; the EBNA-2 gene is directly involved, but additional factors such as environmental mutagens or mitogens (for example, malaria infection) or cytogenic errors are probably also involved.

Immunosuppression is the only stimulus to EBV reactivation that has been well described; immunosuppressed patients shed EBV in the oropharynx, and it is found in saliva (Yao et al. 1991; Alsip et al. 1988; Diaz-Mitoma et al. 1990; Preiksaitis et al. 1992). The *Bam*HI Z EBV replication activator (Zebra) protein is involved in the switch from viral latency to the productive cycle (Mathew et al. 1994).

### 2.3.8 Hairy Leukoplakia

Hairy leukoplakia (HL) is a white lesion in the mouth originally described in individuals infected with HIV. HL has been considered virtually pathognomonic of HIV infection and a good indicator of impending AIDS (Reichart et al., 1989; but see below). The lesions of HL are corrugated or have a shaggy or "hairy" appearance, are usually found on the lateral margins of the tongue, are mostly without symptoms and have no known pre-malignant potential (Greenspan et al. 1984; Greenspan and Green 1989; Green et al. 1989; Scully et al. 1989).

Histological features include hyperparakeratosis, hyperplasia and ballooning of prickle cells with depletion of Langerhan's cells (Daniels et al. 1987; Kanas et al. 1988; Sciubba et al. 1989) and with only a sparse inflammatory cell infiltrate in the lamina propria (Eversole et al. 1986). Ultrastructural features are discussed

elsewhere (BELTON and EVERSOLE 1986; KANAS et al. 1988). Electron-microscopic examination of cytological smears (EPSTEIN et al. 1995) may be diagnostically superior to Papanicolaou (PAP)-stained smears (MIGLIORATI et al. 1993; LUMERMAN et al. 1990; FRAGA-FERNANDEZ and VICANTI-PLAZA 1992).

EBV has been shown to be present in HL. Electron microscopy, immunostaining, and Southern blot and *in situ* hybridization for EBV DNA shows EBV in the upper spinous layers of the epithelium (SCIUBBA et al. 1989; GROSS et al. 1988; SYRJANEN et al. 1988; GREENSPAN et al. 1984, 1985; EVERSOLE et al. 1988; SCIUBBA and SCHWARTZ, 1987; LONING et al. 1987; SUGIHARA et al. 1990; MABRUK et al. 1994, 1995). Viral proteins characteristic of the replicative phase can be found (YOUNG et al. 1991). EBV receptors on the parakeratinised oral mucosae (CORSO et al. 1989), such as on the lateral margin of the tongue, appear to explain the site predilection for HL, but EBV DNA is also present in some sites where no HL is clinically evident (ZHANG et al. 1988; NAHER et al. 1991). The decrease in epithelial Langerhan's cells might be a sequel of, or might predispose to, viral infection (DRIJKONINGEN et al. 1988). EBV regulation, at least in B lymphocytes, is known to be disturbed in HIV infection (BIRX et al. 1986). The exact site of EBV latency in the epithelium is unclear (BECKER et al. 1991; NIEDOBITEK et al. 1991). EBV-1 or EBV-2 or both may be found, and co-infection and recombination are common (WALLING et al. 1992, 1994, 1995). The EBV in patients from the West usually appear to lack EBNA-2 or are present as EBNA-2-defective variants which may escape T cell attack (GILLIGAN et al. 1990; PATTON et al. 1990; WALLING et al. 1994) or may result in the unusual course of this infection. LMP are expressed (GILLIGAN et al. 1990; THOMAS et al. 1991; SANDVEJ et al. 1992).

Further evidence for a causal role for EBV in HL is the regression of HL on treatment with antivirals which block EBV replication, such as acyclovir (FRIEDMAN-KIEN, 1986; USCHENDORF et al. 1988; RESNICK et al. 1988; FICARRA et al. 1988) or ganciclovir (NEWMAN and POLK, 1987), and the failure to resolve with antifungals (FICARRA et al. 1988), despite the frequent presence of candida species. HL in HIV-infected individuals may also occasionally improve spontaneously or with zidovudine (BROCKMEYER et al. 1989; KESSLER et al. 1988; PHELAN and KLEIN, 1988). However, HL is usually without symptoms and, though it may be the source of concern to the patient, its treatment is rarely indicated.

Thus it appears that HL is associated with EBV and is not uncommon in HIV-infected individuals. However, recent work indicates that the situation is more complex than formerly supposed. Firstly, oral white lesions other than HL can occasionally be seen in HIV-infected subjects, and these may have some of the histological features typical of HL, especially the hyperparakeratosis (GREEN et al. 1989; EVERSOLE et al. 1986); however, they are typically EBV DNA negative, though it seems likely that EBV DNA will be found in some, in view of its evident latency in oral and oropharyngeal mucosa (SIXBEY et al. 1984; JALAL et al. 1992; MABRUK et al. 1995). Secondly, it is increasingly evident, and not surprising, that lesions clinically and histologically similar to HL can be seen in patients with HIV infection (BREHMER-ANDERSSON et al. 1994). Finally, HL may be seen in patients immunocompromised for reasons other than HIV infection, such as those undergoing bone marrow (EPSTEIN et al. 1988, 1991, 1993; BIREK et al. 1989), renal (ITIN

et al. 1988; Syrjanen et al. 1989; Greenspan et al. 1989; Macleod et al. 1990; Kanitakis et al. 1991), heart (Schmidt-Westhausen et al. 1990, 1991) or liver transplants (Schmidt-Westhausen et al. 1993), in myelodysplasia (Ficarra et al. 1991), ulcerative colitis (Fluckiger et al. 1994) and Wegener's granulomatosis (Walling et al. 1995) and in patients on systemic corticosteroids (Schiodt et al. 1995). HL has even been seen in apparently healthy HIV-seronegative subjects (Eisenberg et al. 1992), though in others their HIV status could not be absolutely assured (Greenspan and Green, 1989).

Thus it appears that HL is not absolutely specific for HIV, but rather a manifestation of chronic immunosuppression, and it is occasionally seen in apparently immunocompetent persons. In immunocompromised patients, the occurrence of HL is not always related to the CD4 lymphocyte count (Reichart et al. 1989; Schmidt-Westhausen et al. 1993).

HL does not appear to be potentially malignant and the EBNA-2 defect may explain this, since that gene is required for transformation. Nevertheless, there may be an association between HL and the subsequent development of malignant lymphoproliferative diseases (Moore et al. 1991).

### 2.3.9 Lymphoproliferative Diseases

EBV infection can lead to a number of lymphoproliferative diseases in which transformation-associated EBV genes such as LMP-1 can be found (Young et al. 1989).

*2.3.9.1 Burkitt's Lymphoma.* Burkitt's lymphoma (BL) is a highly malignant B lymphocyte lymphoma found endemically in equatorial Africa and in Papua New Guinea. It was the first human tumour known to be associated with a virus. African BL is found in areas where there is hyperendemic malaria, and it accounts for about 50% of childhood malignancies in Africa. Sporadic cases are found elsewhere and in HIV disease.

*2.3.9.1.1 Epidemiology.* BL is virtually unknown in infancy. The peak incidence in African BL is in the 4- to 7-year age-group, and 80% of patients fall in the 3- to 12-year age-group. There is male to female predominance of 2:1 to 4:1. Jaw involvement is most common in the younger age-groups. Most patients in Africa have been African; elsewhere there is no notable racial predilection.

*2.3.9.1.2 Pathogenesis.* EBV was first found in explants of BL following the search for an infectious agent suggested by the unique geographic distribution of African BL. Virus particles are not seen in tumour biopsy material; presumably, tissue culture releases a block that inhibits expression of latent EBV.

The first direct evidence of viral-specific material in these tumours was provided by the discovery of MA on freshly biopsied tumour cells and of EBNA in at least 90% of cells, though EBNA-2 and LMP appear to be down-regulated (Rowe et al. 1987). EBV DNA is almost invariably detectable in biopsy material. These studies demonstrate that the EBV genome is present and expressed in the tumour

cells in African BL. Further evidence of an oncogenic role of the virus is afforded by the demonstration of the ability of EBV to transform B lymphocytes *in vitro* and induce tumours in primates. The titres of serum antibodies directed against EBV antigens are increased in patients with Burkitt's lymphoma; anti-EBNA are present with very high titres of IgG anti-VCA and antibody to membrane antigen (anti-MA). Anti-EA (R) also appears.

However, EBV infection alone is clearly insufficient for tumour production, since many apparently normal individuals are infected with EBV without any malignant sequelae and, in American BL, EBV DNA is usually absent. Genetic, life-style or environmental factors must be involved. Malaria and chromosome abnormalities have been implicated as co-factors. Malaria may influence the immune response to EBV, and such patients may also be immunocompromised secondary to malnutrition. Chromosomal changes are also involved.

The first step in development of the lymphoma seems to be the induction of polyclonal B cell immortalisation by EBV, followed by promotion mediated by environmental factors, such as malaria (which stimulates further proliferation of B cells), and, finally, chronic B cell proliferation. Specific chromosome defects, which appear to occur randomly during the latter stage, result in a reciprocal 8–14 chromosomal translocation, the *myc* oncogene coming to be placed next to the immunoglobulin gene locus and subsequently be stimulated, leading to dysregulation of cell growth resulting in the lymphoma. EBV in B cells appears to elude virus-specific T cell recognition (Rooney et al., 1985).

*2.3.9.1.3 Clinical Features.* BL is typically multifocal, affecting the jaws, bone marrow, post-orbital region and gastro-intestinal tract and, in girls, breasts and ovaries.

Approximately 50% of children with African BL present with jaw tumours, but in non-African cases only about 5% have jaw lesions (Patton et al., 1990). The maxilla is mainly affected, and tumours are mainly in the molar–premolar region. Lesions in the mandible develop in the posterior body. Some 40% have multiple jaw tumours.

Dental findings include sudden, painless loosening of teeth, premature tooth eruption or displacement of teeth. Interestingly, labial anaesthesia is rare, though cranial nerve palsies due to intracranial disease are common. Radiography shows breaks in, or loss of the lamina dura, small discrete jaw radiolucencies and possibly widening of the periodontal ligament space. However, root resorption is rare (Hopp et al. 1982).

Salivary gland involvement is seen clinically in about 4% of patients, but three times as many have histological involvement. Cervical lymphadenopathy is common (Akinwande et al. 1986; Mosadomi 1984).

*2.3.9.1.4 Diagnosis.* The clinical features of BL in a patient from an endemic area are usually suggestive, but a biopsy is needed. The histological appearance is of a poorly differentiated lymphocytic lymphoma composed mainly of surface IgM-positive B cells. Large lymphocytes with irregularly shaped (cleaved) nucleoli and cytoplasm confined to the cell periphery are present, with scattered, large, pale-staining macrophages giving a distinctive "starry sky" appearance.

*2.3.9.1.5 Management.* BL is sensitive to cyclophosphamide and, though a single dose may be sufficient to cause tumour regression, this drug is often used in combination with vincristine, methotrexate and corticosteroids. Local surgery or radiotherapy may help debulk a tumour. Survival rates exceed 50% and are best in younger children treated early and having minimal tumour burden.

*2.3.9.2 Non-Hodgkin's Lymphomas.* Non-Hodgkin's lymphomas (NHL) are being increasingly seen since the advent of the AIDS epidemic (Karp and Broder, 1991). Approximately 3% of HIV-infected persons have NHL at the onset of AIDS, and projections suggest that many more will later develop NHL (Gail et al. 1991). Indeed, one autopsy study showed NHL in 20% of HIV-infected patients (Wilkes et al. 1988). Lymphomas have emerged as an increasingly common cause of death in AIDS; patients often present at an advanced stage, with extranodal involvement, and respond poorly to chemotherapy (Myskowski et al. 1990; Carbone et al. 1991).

EBV is associated with about one half of the NHL in HIV disease (Lenoir and Delecluse, 1989). The onset of lymphomas in HIV disease is preceded by persistent generalised lymphadenopathy (PGL) in one third of cases, and therefore enlargment of pre-existent palpable lymph nodes is always an indication for a biopsy to exclude malignant lymphoma. Most NHL in AIDS are high-grade B cell malignancies, particularly diffuse large cell lymphomas and immunoblastic lymphomas, but primary lymphomas of the brain, especially BL, are also common (Beral et al. 1991; Carbone et al. 1991). At initial presentation, the lymphomas in AIDS are typically widely disseminated, with extranodal involvement in 65%–98% of patients. The CNS is commonly involved, presenting either as leptomeningeal lymphoma in patients with systemic disease or primary HIV-related lymphoma in the CNS, and gastro-intestinal tract involvement is seen in 7%–45% (Ziegler et al. 1984; Karp and Broder 1991).

Oral NHL are now a recognised, but uncommon complication of HIV infection, typically observed as a rapidly growing mass in the fauces, gingiva or elsewhere, as an ulcer or as tooth mobility (Ziegler et al. 1984; Lozada-Nur et al. 1984; Leess et al. 1987; Hommel et al. 1987; Brahim et al. 1988; Kaugars and Burns, 1989; Green and Eversole, 1989; Rubin et al. 1989; Mitchell et al. 1989; Soderholm et al. 1990; Groot et al. 1990; Colmenero et al. 1991; Carbone et al. 1991; Langford et al. 1991; Donkor et al. 1991). Oral lymphomas in HIV disease may be associated with EBV (Green and Eversole, 1989).

*2.3.9.3 Other Diseases*

*2.3.9.3.1 Mid-line Granuloma.* EBV has been associated with mid-line granuloma (Vilde et al. 1985; Harabuchi et al. 1990), now known to be a lymphoma.

*2.3.9.3.2 Hodgkin's Disease.* Hodgkin's disease (HD) occurs in two peaks, at around age 25 years and at around 70 years, the younger type being found in lower socio-economic conditions. EBV serum antibody titres are higher in lymphocyte depletion and mixed cellularity types of HD, and EBV RNA and LMP have now been detected in some HD (Young et al. 1989).

*2.3.9.3.3 Duncan's Disease.* Patients with the X-linked lymphoproliferative syndrome (Duncan's disease), if exposed to EBV, may develop a spectrum of disorders ranging from fatal infectious mononucleosis to acquired hypogammaglobulinaemia following infectious mononucleosis, or lymphoma (PURTILO 1980; PURTILO and SAKAMOTO 1981; PURTILO et al. 1982; SAEMUNDSEN et al. 1981).

*2.3.9.3.4 Lymphomatoid Granulomatosis.* Lymphomatoid granulomatosis is an unusual entity which is characterised by a mixed mononuclear cell infiltrate and areas of necrosis with atypical lymphoreticular cells and which, in some 20%–50% of cases, progresses to malignant lymphoma.

Several cases of lymphomatoid granulomatosis have been seen in AIDS patients as oral ulcers (MONTILLA et al. 1987; LIN-GREENBERG et al. 1990). EBV infection has been implicated in HIV-related lymphomatoid granulomatosis (MITTAL et al. 1990).

*2.3.9.3.5 Thymic Lymphoma.* EBV may be involved in thymic lymphoma (LEYVRAZ et al. 1985; DIMERY et al. 1988).

*2.3.9.3.6 Hairy Cell Leukaemia.* EBV has also been implicated in hairy cell leukaemia, a rare form of leukaemia (WOLF et al. 1990).

*2.3.9.3.7 Sjogren's Syndrome.* Sjogren's syndrome (SS) is a B cell lymphoproliferative disorder that may lead to lymphoma and has occasionally closely followed primary EBV infection (WHITTINGHAM et al. 1985) PFUGFELDER et al. 1987; GASTON et al. 1991), suggesting that EBV may be one factor initiating SS. The possible association of EBV with SS is discussed elsewhere (MAITLAND and SCULLY, 1994; MIYASAKA et al. 1994). EBV RNA may be associated with the auto-antigens SS-A (Ro) and SS-B (La) found in SS (LERNER et al. 1981). EBV DNA and EBV EA may be found in SS-affected salivary tissue (MARIETTE et al. 1991; SAITO et al. 1989; KARAMERIS et al. 1992; DEACON et al. 1991, 1992; MAITLAND et al. 1995; DISS et al. 1995), though others have not found EBV EA (SYRJANEN et al. 1990). EBV DNA appears to be found in salivary glands in amounts greater than in other auto-immune diseases or normal salivary glands in some studies (MARIETTE et al. 1991; SCHUURMAN et al. 1989; SAITO et al. 1989; KARAMERIS et al. 1992), but not in others (DEACON 1991, 1992; MAITLAND et al. 1995). The detection of EBV DNA, however, appears dependent on the methodology, with, for example, PCR detecting EBV DNA in some samples which appear EBV negative by *in situ* hybridisation (MARIETTE et al. 1991; SYRJANEN et al. 1990).

The salivary glands are therefore a possible site of latency of EBV (and other viruses), although the findings neither confirm nor refute a direct association of HCMV or EBV with SS. It may be that the clinical picture termed SS is the common end-result of various aetiological factors and that different viruses might be a trigger in genetically susceptible patients or may simply be reactivated and non-causal (FOX et al., 1991). Indeed, a wide range of viruses, including HCMV, EBV, hepatitis C virus and, more recently, various retroviruses, have been implicated in SS. A salivary gland syndrome resembling SS has also been described in HIV

patients (SCHIODT et al. 1989) and in those infected with HTLV-1 (SHATTLES et al. 1992; TERADA et al. 1994; SUMIDA et al. 1994).

Thus, although EBV and several other herpesviruses may latently infect salivary glands, any individual or collective role for these viruses or other agents singly or multiply in the disease remains to be confirmed.

*2.3.9.3.8 Salivary Gland Tumours.* EBV appears unrelated to the pathogenesis of most salivary gland neoplasms, with the possible exception of lymphoepithelial carcinoma (RAAB-TRAUB et al. 1991; HSU et al. 1994), though EBV DNA was found in all five salivary gland tumours (undefined) in one study (TYAN et al. 1993).

## 2.3.10 Carcinomas

*2.3.10.1 Nasopharyngeal Carcinoma.* EBV is also associated with undifferentiated nasopharyngeal carcinomas (FREEMAN et al. 1994; NASRIN et al. 1994), though environmental factors (probably nitrosamines from smoked fish), and genetic factors are important. There is familial clustering of cases and an association with certain HLA haplotypes in Chinese patients, particularly A2 and the antigen Singapore-2. However, no association with HLA types has been demonstrated in Tunisian patients with nasopharyngeal carcinoma. In causasoids, HLA-A2 appears to be protective (BURT et al. 1994).

The serology of nasopharyngeal carcinoma is somewhat similar to that of BL, with very high titres of IgG anti-VCA and anti-MA, but with high anti-EBNA and the presence of IgA anti-VCA. Anti-VCA and anti-EA of the IgA class may be predictive of the development of nasopharyngeal carcinoma. Furthermore, the anti-EA of nasopharyngeal carcinoma patients is of a different type (anti-D) from that found in BL (anti-R).

EBV DNA has repeatedly been shown to be present in tumour tissue from nasopharyngeal carcinoma and is also found in tumours from widely differing geographical areas, but only in undifferentiated anaplastic tumours, in the epithelium rather than in lymphoid tissues. EBNA-1, LMP-1 and LMP-2A/2B have also been detected (YOUNG et al. 1988; LIEBOWITZ 1994).

*2.3.10.2 Oral Carcinoma.* The association of EBV with anaplastic nasopharyngeal carcinoma is well established, and the oncogenicity of EBV is not in doubt. EBV DNA has been detected in oral carcinomas by some (MAO and SMITH, 1993; TYAN et al. 1993; HORIUCHI et al., 1995), but EBV DNA and antigens have not been demonstrated by others in oral carcinoma tissue or in carcinoma cell lines (KARJA et al. 1988; TALACKO et al. 1991; YIN et al. 1991).

*2.3.10.3 Antral Carcinoma.* In one very small series of three tumours, all contained EBV DNA (TYAN et al. 1993).

*2.3.10.4 Adult Rhabdomyoma.* EBV has been discounted in the aetiology of adult rhabdomyoma (CLEVELAND et al. 1994).

*2.3.10.5 Aphthae.* EBV appears unrelated to the aetiology of aphthae (PEDERSEN et al. 1993).

## 2.4 Human Cytomegalovirus

HCMV is a ubiquitous herpesvirus. It is emerging as an important opportunistic pathogen in immunocompromised individuals, particularly those infected with HIV; it is now apparent that HCMV may cause oral ulceration in immunocompromised subjects, and it may play a role in other diverse conditions in these and other patients (FORBES 1989; EPSTEIN and SCULLY 1994).

Sometimes termed human herpesvirus-5, or beta herpesvirus, HCMV is indistinguishable by electron microscopy from HSV and VZV. There are several strains of HCMV, but these do not appear distinct to particular syndromes (SCULLY and SAMARANAYAKE 1992; EPSTEIN and SCULLY 1994; SCULLY 1995).

### 2.4.1 Epidemiology

HCMV infection is much more common than identifiable clinical disease, with serological evidence of previous infection in up to 80% by adult life (BELSHE 1984; BERRY et al. 1988; Ho, 1990). HCMV infection is endemic worldwide. In developing countries, HCMV infection is usually acquired in early childhood, but in the developed world many escape infection in childhood, and then sexual transmission plays a greater role. In the United Kingdom, by 35 years of age about 50% of the population have antibody to HCMV (BELSHE 1984; BERRY et al. 1988; FORBES 1989; Ho, 1990).

HCMV is readily transmitted in infected blood and tissues and can be transmitted sexually (EPSTEIN and SCULLY, 1994; SPECTOR et al. 1984; ADLER et al. 1982). Maternal antibodies appear not to protect infants, who can be infected via genital HCMV or virus in breast milk (STAGNO et al. 1980).

### 2.4.2 Immunopathogenesis

HCMV can infect all nucleated cells and, after infection, these cells express IE and E HCMV antigens and can then become the target for cytotoxic T lymphocytes. HCMV infects salivary acinar cells, and ductal epithelial cells are especially infected, particularly those in the major salivary glands and proximal renal tubules. HCMV appears to induce a series of cellular responses that characteristically cause cell enlargement (*cytomegalo*) with intranuclear inclusions; hence the formerly used term, cytomegalic inclusion disease (ALBRECHT et al. 1990). HCMV also infects phagocytes and via these and other routes can spread throughout the body.

T lymphocytes play a major role in immune surveillance; CD8 and, in salivary glands CD4 cells, are protective in concert with $\gamma$-interferon (LUCIN et al. 1992). Humoral immune responses to HCMV may also to some extent be protective. IgM-

specific antibodies appear early in HCMV infection, peak at 2–4 weeks and are usually undetectable within 16 weeks. IgG antibodies peak at 1–2 months. Immunocompromised subjects who have defective cellular immunity and no IgM anti-HCMV antibodies are at risk of disseminated HCMV infections.

A gene similar to that coding cellular MHC class 1 antigens is found in HCMV, and this may be involved in viral attachment and may also interfere with immune responses (Wiley 1988; Griffiths and Grundy 1988). HCMV can probably also interfere with complement activation, antigen recognition and inflammatory cell function (Banks and Rouse 1992). HCMV is immunosuppressive (Rook 1988) and thus, like other herpesviruses, may be able to modulate the course of other infections, such as HIV.

### 2.4.3 Clinical Features of Primary Infection

Most primary infections with HCMV are asymptomatic or cause only mild, flu-like symptoms, but over 90% of those infected have subclinical hepatitis. Infection of otherwise healthy young infants rarely causes obvious clinical illness, but infection in older children may manifest with anicteric hepatitis, respiratory disease or a mononucleosis-like syndrome with a negative heterophile antibody test (Paul-Bunnell test) and little in the way of tonsillitis, lymphadenopathy or splenomegaly. Otherwise healthy adolescents and adults can also present with infection which, after an incubation of 4–8 weeks, appears as a glandular fever-like syndrome that is Paul-Bunnell negative, or they may have blood dyscrasias (usually thrombocytopenia or haemolytic anaemis) or, rarely, pneumonia or encephalitis (Belshe 1984; Ho, 1984; Scully and Samaranayake 1992; Epstein and Scully 1994).

### 2.4.4 Latency

HCMV remains latent after the primary infection mainly in oropharyngeal and renal epithelial cells. Lymphocytes and monocytes have also been implicated as sites of latency (Ho 1982), and there may, in fact, be multiple sites of latency. HCMV may be harboured with no ill effects, but reactivation is common, with viral shedding, and disease may result, especially in immunocompromised subjects. HCMV appears in the blood, oropharyngeal secretions, saliva, urine, breast milk, tears, sputum, faeces and genital fluids of infected individuals (Epstein and Scully 1994), and there may be chronic shedding of the virus, particularly from immunocompromised subjects.

### 2.4.5 Infection in Pregnancy

The fetus is susceptible at any stage of pregnancy and, though HCMV infection or reactivation in the mother is often asymptomatic, HCMV infection may be transmitted to the foetus and produces damage (Peckham 1991; Stagno and

WHITLEY 1985). Indeed, HCMV now causes more congenital abnormalities and mental handicap in Western countries than does rubella, though only 0.5% live births are infected and only 5%–10% of HCMV-infected fetuses are severely damaged (PECKHAM 1991; BEST 1987; STAGNO and WHITLEY 1985).

The resultant clinical picture in an HCMV-infected fetus may include low birth weight, prematurity, purpura, anaemia, jaundice, microcephaly/hydrocephaly, cerebral calcification, cataracts, chorioretinitis, micro-ophthalmia and pneumonitis. Liver disease, microcephaly and mental handicap are the main defects. There may incidentally also be hypoplasia in the deciduous dentition. A further 10%–15% of those infected but without obvious abnormalities at birth may later show hearing loss or a degree of mental handicap (STAGNO and WHITLEY 1985).

Lymphocytes from mothers in whom HCMV infects the fetus appear to be impaired in their *in vitro* responses to HCMV antigens (OKABE et al. 1983; PASS et al. 1983).

### 2.4.6 Infection in Immunocompromised Patients

Primary or reactivated HCMV infections are extremely common in immunocompromised patients, especially bone marrow or organ transplant patients and those with HIV disease (JACOBSEN and MILLS 1988; SUTTMANN et al., 1988). HCMV is also the most common viral infectious complication in patients after tissue transplantation and is a cause of significant morbidity and mortality in many immunocompromised patients (BELSHE 1984; HO, 1982; BERRY et al. 1988; SPECTOR et al. 1984; JACOBSEN and MILLS 1988).

*2.4.6.1 Bone Marrow Transplantation.* HCMV reactivation or re-infection is seen in more than 75% of HCMV-seropositive patients who receive bone marrow transplants (WINSTON et al. 1982; HERSMAN et al. 1982; MEYERS and THOMAS 1988). The incidence of serious infection is even higher if the transplant recipients are HCMV seronegative but their donor seropositive, i.e. where there is primary HCMV infection in the bone marrow recipient (HERSMAN et al. 1982; MEYERS and THOMAS 1988).

Together with HSV, HCMV is implicated in about one quarter of cases of oesophagitis following bone marrow transplantation and, with the control of HSV by acyclovir, HCMV infections may be expected to increase in proportion (MEYERS and THOMAS 1988). Ulcerations due to HCMV have now been identified at all levels in the gastro-intestinal tract, including the oral cavity, as discussed below.

*2.4.6.2 Organ Transplantation.* There is HCMV infection in up to 96% of patients having solid organ transplantation such as renal, liver or cardiac transplants (RUBIN and TOLKOFF-RUBIN 1984; GENTRY and ZELUFF 1988; RUBIN 1988), usually within the first 4 months after transplantation (GENTRY and ZELUFF 1988; RUBIN 1988).

HCMV infection may produce a mononucleosis syndrome and/or progressive multi-system disease and, following cardiac transplantation, it is the single most common cause of morbidity and mortality, usually from pneumonia (GENTRY and ZELUFF 1988).

*2.4.6.3 Human Immunodeficiency Virus Disease.* Patients with HIV disease are often infected with HCMV and carry several strains of HCMV (SPECTOR et al. 1984; JACOBSEN and MILLS 1988; DREW et al. 1982; LEPORT et al. 1987; POLK et al. 1987), including in their saliva (LUCHT et al. 1993). HCMV infection may also lead to a range of clinical manifestations from a mononucleosis syndrome (LEPORT et al. 1987), to pneumonitis, hepatitis, chorioretinitis, CNS infection (POLK et al. 1987; GOTTLIEB et al., 1981; LERNER and TAPPER 1984) and gastro-intestinal ulceration (including oral ulcers; see later), and may be lethal (MACHER et al. 1983). Indeed, HCMV infection is a predictor of AIDS in those infected with HIV (POLK et al. 1987).

HCMV appears to assume increased pathogenicity in HIV disease and may interact with HIV (HIRSCH et al. 1984), as well as exacerbating the immune defect, like many herpesvirus infections (MERIGAN 1981), causing suppression of NK cell and cytotoxic T lymphocyte function.

Thus there are good theoretical and practical reasons for attempting to prevent and for treating HCMV infections in immunocompromised persons.

*2.4.6.4 Gastro-intestinal and Oral Ulceration Related to HCMV Infection in Immunocompromised Patients.* Oesophageal ulcers have been the principle clinical lesions of infection with HCMV in some immunocompromised individuals (TOGHILL and MCGAUGHEY 1972; ST. ONGE and BEZAHLER 1982; VILLAR et al. 1984; MYERSON et al. 1984; RABENECK et al. 1990) and have been the presenting feature in some HIV-infected subjects (RABENECK et al. 1990). The ulceration appears to result from vasculitis (RUBIN 1988). Diagnostic criteria have varied, but ulcerations have usually been attributed to HCMV when viral inclusions have been present in cells in adjacent connective tissue and HCMV has been isolated from the lesions.

HCMV-related oral ulcers have also been reported in immunocompromised patients (MYERSON et al. 1984; JONES et al. 1993), sometimes with underlying osteomyelitis (JONES et al. 1993; BERMAN and JENSEN 1990). Inclusions suggestive of HCMV infection may be seen in the endothelium subjacent to the ulcers, although in these cases no direct infection of the epithelium by HCMV has been proved.

Pharyngeal (LALWANI and SNYDERMAN 1991) and oral ulcerations (ANDRIOLO et al. 1986; KANAS et al. 1987; CLICK et al. 1991; LANGFORD et al. 1990; JONES et al. 1992, 1993; SCHUBERT et al. 1993) due to HCMV have also now been described in patients with HIV disease, the diagnosis of HCMV usually being based upon light or electron microscopic demonstration of intranuclear and cytoplasmic HCMV inclusions. HCMV inclusions are eosinophilic, and intracytoplasmic inclusions stain with Gomori's methenamine silver or with periodic acid–Schiff.

In four cases of HCMV-associated oral ulcers in HIV-infected patients who had disseminated HCMV infection, tissue HCMV inclusions were seen on light microscopy and the diagnosis was confirmed by immunohistochemistry and in situ DNA hybridisation. HCMV was detected principally in endothelial cells or perivascular and subepithelial connective tissue. HCMV antigen was detected in occasional epithelial cells. Activated T lymphocytes were identified in the tissue, and high titres of serum HCMV antibodies were present (LANGFORD et al. 1990). In other cases, HCMV was also confirmed immunohistochemically or by in situ DNA hybridisation in some cases (JONES et al. 1992, 1993).

It is tempting to speculate that some other aphthous-type oral ulcers disease may be HCMV related, but this needs further investigation (see below).

### 2.4.7 Diagnosis

Serology and a full blood picture are usually indicated, inorder to exclude other causes of glandular fever-like syndromes, especially EBV or HIV. Enzyme-linked immunosorbent assay (ELISA) for IgM HCMV is the most widely accepted specific diagnostic assay at present (ROOK, 1988) but, in practice, serology is currently under-used for specific diagnosis (SCULLY and SAMARANAYAKE 1992; EPSTEIN and SCULLY 1994; BELSHE 1984; HO 1982).

Viral culture of body fluids or tissues has been the "gold standard" for diagnosis of infection. However, as viral shedding may occur in the absence of clinical disease, it may not necessarily indicate a pathogenic role for HCMV. Direct techniques in tissue studies *may be* more specific. Tissue biopsy may show the typical histopathological changes of HCMV infection, with enlarged cells and inclusion bodies (Cowdry type A) in nuclei and cytoplasm. However, biopsy is not usually indicated, and it is more reasonable to examine for HCMV antigens in infected cells or secretions by immunostaining using specific monoclonal antibodies such as in detection of EA fluorescent foci (DEAFF) or the leucocyte antigen detection (LAD) test. HCMV nucleic acids can also be sought by *in situ*, dot blot or Southern blot hybridization or by PCR, techniques now increasingly used for diagnosis (MANGANO et al., 1992; WARREN et al., 1992; SHUSTER and BENEKE, 1985). PCR, in particular, is proving extremely useful (WARREN et al. 1992; GREENBERG et al. 1995) and can facilitate rapid diagnosis.

### 2.4.8 Possible Associations with Other Conditions

*2.4.8.1 Kaposi's Sarcoma.* Epidemiological data – particularly the high prevalence of Kaposi's sarcoma (KS) in homosexuals and reports of cases of KS in homosexuals who were not HIV antibody positive after 8–10 years – suggested that KS in HIV disease might be associated with a transmissible agent other than HIV (ARCHER et al. 1989; BERAL et al. 1990; FRIEDMAN-KIEN et al. 1990; HAVERKOS et al. 1990; MARQUART et al. 1991). HCMV was one candidate, but it may only have been an opportunistic infection within the KS tissue, as may mycoplasma or other agents

(MARQUART and OEHLSCHLAEGEL 1985; LO et al. 1989). HCMV can be latent in endothelium even in non-HIV-infected persons and may thus be an incidental finding in the lesions of KS (BELSHE 1984; HO 1982).

Recent findings of a novel herpesviruses in KS further discount a role for HCMV (see below).

*2.4.8.2 Oral Carcinoma.* There is, at least on serological evidence, no association between HCMV and oral carcinoma (Larsson et al. 1991).

*2.4.8.3 Salivary Gland Disease.* HCMV is an interesting virus because it was first recognised in salivary glands as the "salivary gland inclusion virus". Nevertheless, it has not been proved to cause salivary gland disease. It has been reported that salivary gland tumours can be induced experimentally by mouse CMV (LAMEY et al., 1982). CMV has induced adenocarcinomas in mice, but several of the animals had been irradiated or received anti-lymphocytic serum, and thus immune or other factors may have been at play. HCMV is also a possible candidate virus for involvement in human salivary gland tumours, though HCMV has not been cultured from salivary tumours (LAMEY et al. 1982) and HCMV antigens are not found (FOX et al. 1986), but, again, this does not necessarily exclude involvement. HCMV DNA could be present without antigen expression.

HCMV may also be implicated in SS. Some serological studies have shown normal serum titres of HCMV antibodies in SS (SCULLY 1989), but others have shown raised titres (SHILLITOE and ALSPAUGH 1985; THOM et al. 1988), suggesting there may be viral reactivation. "Virus-like" structures have been seen by electron microscopy in minor salivary glands and other tissues in SS, but have not been shown to be viral, and immunostaining has failed to demonstrate HCMV antigens. These structures may be associated in some way with the abnormal immunoglobulin synthesis seen in SS.

HCMV DNA has been found by us in SS salivary tissue, but it is also found in non-specific sialadenitis (MAITLAND et al. 1995). Furthermore, the lymphomas complicating SS appear not to contain HCMV DNA (FOX 1989). An aetiological link between HCMV and SS therefore has little support, despite suggestions of some viral link (FLESCHER and TALAL 1991).

HCMV might also be implicated in xerostomia in HIV disease, and although one study showed no relation (LUCHT et al. 1993), there was a strong correlation between the presence of HCMV in saliva and xerostomia in another study (GREENBERG et al. 1995), and HCMV has also been implicated in submandibular sialadenitis, which was responsive to ganciclovir in one patient with AIDS (PIALOUX et al. 1991).

*2.4.8.4 Behcet's Syndrome and Aphthae.* There may be reactivation of HCMV, but there are no consistent antibody responses to HCMV in aphthae (PEDERSEN 1989; PEDERSEN and HORNSLETH 1993); HCMV is not detectable in lesions (OGAWA et al. 1990) and, in Behcet's syndrome, HCMV serum antibodies are not increased (HAMZAOUI et al., 1990), making a regular association between HCMV and these

conditions unlikely. However, perhaps up to 10% of oral ulcers contain HCMV (Leimola-Virtanen et al. 1995).

*2.4.8.5 Graft Versus Host Disease.* The possible association between HCMV and graft versus host disease (GVHD) is discussed at length elsewhere (Appleton and Suiland 1993).

### 2.4.9 Prophylaxis and Treatment

Passive immunisation using specific immunoglobulin with high-titre, anti-HCMV antibody may provide a degree of protection against primary infection in seronegative subjects who are accidentally exposed to HCMV. No reliably effective vaccine is available against HCMV.

Unfortunately, acyclovir is not reliably effective in treating active HCMV infection (Meyers 1989; Selby et al. 1989), presumably because HCMV lacks thymidine kinase (Meyers 1989). Acyclovir may have some activity against HCMV reactivation. Low doses of acyclovir – 250 mg/m$^2$ t.i.d. (Saral et al. 1981) or 5 mg/kg b.i.d. (Hann et al. 1983) – have not, however, proved reliably effective in prevention of HCMV reactivation after bone marrow transplantation, though one study reported that oral acyclovir (200 mg, q.i.d.) significantly reduced HCMV shedding (Gluckman et al. 1983) and high-dose acyclovir (450 mg/m$^2$, p.i.d.) prevented HCMV reactivation (Meyers et al. 1988).

The principle anti-viral against HCMV is ganciclovir (2-hydroxy-1-(hydroxymethyl)-ethoxymethyl guanine) (DHPG) (Balfour and Englund 1989; Field et al. 1983; De Clerc 1988). Unfortunately, bone marrow suppression results in up to 50% of those treated with ganciclovir (Hann et al. 1983; Balfour and Englund 1989; Reed et al. 1990), and there may also be other adverse effects, such as fever, rashes, nausea, vomiting, CNS toxicity and hepatotoxicity. Ganciclovir is therefore indicated mainly for serious HCMV infections (Laskin et al. 1987) such as retinitis. Ganciclovir is effective treatment for HCMV retinitis in AIDS (Bryson 1988) and for HCMV pneumonitis in solid organ transplantation (Mai et al. 1989), but not in bone marrow transplantation patients (Reed et al. 1990).

Unfortunately, HCMV may become ganciclovir resistant (Erice et al. 1989). Foscarnet (phosphonoformate) inhibits HCMV DNA polymerase, but, unfortunately, renal toxicity is seen in up to half of the patients (Bryson, 1988; Farese et al. 1990), and nausea, malaise, vomiting, fatigue, headache, other CNS toxicity, haematologic toxicity and hepatotoxicity may be seen. Foscarnet is nevertheless currently the only really effective treatment for ganciclovir-resistant HCMV infections (Scully 1995).

## 2.5 Human Herpesvirus-6

Human herpesvirus-6 was initially identified a decade ago and thought to be a human B cell lymphotropic virus (HBLV) restricted to lymphoproliferative disor-

ders (Salahuddin et al. 1986). It is now known to be the causal agent of a rash, exanthem subitum (Yamanishi et al. 1988; Ueda et al. 1989), and may be associated with GVHD (Yoshikawa et al. 1991). Most children develop antibody before the age of 1 year, indicative of previous infection (Takashashi et al. 1988). Two variants of human herpesvirus-6, A and B, have been identified (Ablashi et al. 1993).

Human herpesvirus-6 is commonly found in saliva (Pietroboni et al. 1988; Fox et al. 1990; Gopal et al. 1990; Harnett et al. 1990; Kido et al. 1990; Levy et al. 1990), especially in immunocompromised patients, but it is not known to be associated with aphthae (Pedersen et al. 1993) or any specific oral disease. One recent study has indicated a high prevalence of serum antibodies to human herpesvirus-6 in patients with oral carcinoma compared with controls, though the significance of these observations is unclear and the findings are not specific (Vasudevan et al. 1991).

Finally, this is another sialotropic herpesvirus that should be considered in SS (Krueger et al. 1990). Raised serum antibody levels to human herpesvirus-6 have been found in SS (Ablashi et al. 1989; Biberfeld et al. 1988), and human herpesvirus-6 DNA has been found in salivary glands (Krueger et al. 1990; Haddad et al. 1992). It has also been detected in lymphomas in SS (Fox et al. 1990; Ablashi et al. 1991). However, causal relationships have yet to be established.

## 2.6 Human Herpesvirus-7

Human herpesvirus-7 resembles human herpesvirus-6, but is a separate entity (Frenkel et al. 1990). It is found in saliva of children and adults, rarely of infants (Hidaka et al. 1993; Black et al. 1993). Infection appears to be typically acquired between the ages of 2 and 5 years (Wyatt et al. 1991). A pathogenic role remains to be established.

## 2.7 Kaposi's Sarcoma Herpesvirus (Human Herpesvirus-8)

Herpesvirus-like DNA sequences resembling, but distinct from, EBV have been demonstrated in tissue from KS of the endemic, sporadic and AIDS-related types (Chang 1994; Schalling et al. 1995), from skin carcinoma in immunosuppressed patients (Rady et al. 1995) and from abdominal B cell lymphomas in AIDS (Cesarman et al. 1995). Tentatively termed KS herpesvirus (KSHV), this agent has been found in all lesions and from all sites studied and has been found in blood but not throat swabs; it was not found in non-KS tissue (Su et al. 1995; Huang et al. 1995; Ambroziak et al. 1995; Moore and Chang 1995; Boshoff et al. 1995; Dupin et al. 1995; Collandre et al. 1995). Whether this agent is present in other disorders is, as yet, unclear (Levy et al. 1990).

## 3 Papillomaviruses

Papillomaviruses are epitheliotropic DNA viruses that have early genes E1–E7, late genes L1 and L2 and a long control region, the upstream regulatory region (URR). They can induce hyperplastic, papillomatous and verrucous lesions in the stratified squamous epithelia of skin and mucosae in a wide range of hosts. Interest in them has been reawakened because of a possible malignant potential, including in relation to oral carcinoma, and because DNA technology has allowed for studies (CHANG et al. 1991; SCULLY et al. 1985, 1988; DE VILLIERS, 1989a; SCULLY and SAMARANAYAKE 1992; SCULLY 1995; SYRJANEN and SCULLY, in press).

Papillomaviruses are classified according to their host range and the relatedness of their nucleic acids, and each is first named according to its natural host, e.g. cottontail rabbit (or Shope) papillomaviruses (CRPV), bovine papillomaviruses (BPV) and human papillomaviruses (HPV).

Papillomaviruses isolated from the same species are subclassified into papillomavirus types according to their nucleotide sequence homology. Any new isolate which has less than 50% cross-hybridisation to previously typed viruses by reassociation kinetics is designated as a new type and numbered in order of discovery. However, if the nucleotide homology exceeds 50%, the virus is considered as a subtype, and if it is close to 100%, with only a few nucleotide differences, it is considered as a variant of the same viral type. More recently, the definition of a new virus type has been modified to rely on the nucleotide sequence of specific viral genes, namely E6, L1 and URR.

### 3.1 Human Papillomavirus Infection

HPV comprise the largest group of papillomaviruses, 73 types had been identified by 1995 (DE VILLIERS 1989a; ZUR HAUSEN and DE VILLIERS 1994). The Papillomavirus Nomenclature Committee agreed at the 1991 Papillomavirus Workshop that, for a novel HPV isolate to be recognised as a new HPV type, its entire genome must be cloned and the nucleotide sequence of the E6, L1 and URR genes should demonstrate less than 90% nucleotide sequence identity with established papillomavirus types.

HPV gain access by direct implantation through breaches in the epithelium. They are shed in cells from the superficial epithelium. HPV induce lesions in many body sites, including the skin and mucosae of the mouth, urethra, larynx, trachea, bronchus, nasal cavity/paranasal sinuses, oesophagus, ano-genital tract, urethra and conjunctivae (CHANG et al. 1992; BRANDSMA and ABRAMSON 1989; YOUNG et al. 1989). Perhaps as importantly, HPV DNA has also been recently demonstrated in *normal* tissues adjacent to HPV lesions in the genital and upper aerodigestive tract, in normal genital mucosa (YOUNG et al. 1989), newborn foreskin and 'by us and others' in normal oral mucosa (see below).

Early studies sought HPV antigens, but HPV are now detected in lesions mainly by examination for HPV DNA (SYRJANEN 1990; SCULLY and SAMARANAYAKE 1992). PCR is the most sensitive test, while Southern blot hybridisation is the most specific detection method.

HPV are now recognised to fall into several general groups. HPV-1, HPV-2, HPV-3 and HPV-4 are seen mainly in cutaneous warty lesions. Another group, comprising HPV-5, HPV-8, HPV-10, HPV-12, HPV-14 and HPV-17, is associated with the unusual cutaneous disorder epidermolysis verruciformis, a disease in which patients have life-long warts, which in some instances, especially in lesions exposed to ultraviolet light, transform to carcinomas. Another group, comprising HPV-6, HPV-11, HPV-16, HPV-18, HPV-31, HPV-35 and others (see below), is associated with mucosal warts or carcinomas, especially in the ano-genital region. Of these, HPV-6 and HPV-11 only have a low risk of association with carcinoma Finally some HPV have very restricted and specific associations for example, HPV-13 and HPV-32 have only been found in oral lesions.

### 3.1.1 Ano-genital Infections

Infections with "genital" HPV types (mainly HPV-6, -11 and -16) appear to be widespread, but are often clinically inapparent and are sometimes transmitted transplacentally, at birth (SEDLACEK et al. 1989; TSENG et al. 1992), sexually (LEY et al. 1991; MURETTO and FERENCZY 1992), by auto-inoculation or by other unidentified routes (PAO et al. 1992). Up to 35% of subjects up to 40 years of age shed HPV ano-genitally (SCHIFFMAN 1994).

Viral DNA is found in over 90% of cervical carcinomas. Convincing experimental data support the concept that specific "high-risk" types of HPV (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52 and -56) are causally involved in the pathogenesis of ano-genital cancer, particularly cancer of the cervix (SHILLITOE 1991; SYRJANEN and SCULLY, in press).

### 3.1.2 Latent and Subclinical Oral Infections

By Southern blot hybridisation, from 15.1% (KELLOWSKI et al. 1992) to over 40% (MAITLAND et al., 1989) of biopsies from clinically normal oral mucosa from adults can be shown to contain HPV DNA, and the PCR technique has increased the detection of HPV DNA in the former study to 21.8% (KELLOWSKI et al., 1992a). HPV DNA can also be shown by PCR in exfoliated normal oral squamous cells (JALAL et al. 1992).

It is clear, therefore, that HPV DNA can be present in normal oral tissues in up to one third or more of the population, and the evidence suggests that the mucosa may act as a reservoir for new HPV infections and/or as a source of recurring HPV lesions. The source of infection is unclear. A study on oral HPV infection in women with past or present genital HPV infection (KELLOWSKI et al. 1990, 1992b) using dot blot hybridisation on exfoliated oral squames showed only a 3.8% HPV DNA prevalence, almost certainly an underestimation

of the true HPV prevalence because basal layer cells cannot be collected in this way.

The term subclinical papillomavirus infection was originally applied to lesions in the uterine cervix that were not visible on clinical inspection but showed histological changes similar to those found in flat warts (SYRJANEN 1989). Though such cervical lesions can be visualised under the colposcope by the application of acetic acid, similar lesions cannot be seen in the mouth (KELLOWSKI et al. 1990). Unfortunately, the term subclinical has been extensively misused in the current literature; it should be reserved exclusively for the epithelial changes, no matter how minor, that do not fulfil the criteria of classical, clinically manifest HPV lesions (SYRJANEN et al. 1989). These epithelial changes are discussed below.

### 3.1.3 Human Papillomavirus-Related Oral Lesions

HPV are clearly implicated in the aetiology of oral squamous cell papillomas, genital warts in the oral cavity (condyloma acuminata), common warts (verrucae vulgaris) in the oral cavity and focal epithelial hyperplasia. HPV have also been demonstrated in a variety of other benign oral lesions, papillary hyperplasia, fibrous hyperplasia, lichen planus and leukoplakia by histopathological, ultrastructural, immunohistochemical and DNA hybridisation studies, though these may not be causal associations (CHANG et al. 1991; SCULLY et al. 1988). Indeed, HPV DNA has also been found in congenital white sponge naevus (COX et al. 1992) and in some odontogenic cysts that have had no apparent connection with the oral cavity (COX et al. 1991).

Among the known HPV types, HPV-1, -2, -4, -6, -7, -11, -13, -16, -18, -32 and -57 have been found in different types of oral lesions. Of these, HPV-13 and HPV-32 seem to be exclusively confined to one specific oral lesion, i.e. focal epithelial hyperplasia (CHANG et al. 1991; SCULLY et al. 1988; DE VILLIERS 1989; GARLICK et al. 1989).

### 3.1.4 Squamous Cell Papilloma

Oral squamous cell papilloma is a relatively common, benign tumour which can occur at any age and is typically found on the palate. HPV aetiology has been proved by showing HPV particles, HPV antigens and HPV DNA, mainly from HPV-6 and HPV-11, in up to 80% of the lesions studied (DE VILLIERS, 1989; YOUNG and MIN 1991). Papillomas may also occasionally contain HPV-2, -13, -16, -32 and HPV-31/33/35 DNA (LONING et al. 1985; NAGHASHFAR et al. 1985; SYRJANEN et al. 1987; EVERSOLE and LAIPIS 1988; YOUNG and MIN 1991).

### 3.1.5 Condyloma Acuminatum

There have been only occasional case reports of oral condyloma acuminatum. They are usually multiple small, white or pink nodules with a surface more cauli-

flower-like than that of papillomas. Differentiation from squamous cell papillomas is difficult and largely academic. Evidence for an HPV aetiology of oral condylomas has been provided by the electron microscopic demonstration of HPV particles, immunohistochemical studies demonstrating HPV antigens and DNA studies showing HPV DNA mainly from HPV-6 and HPV-11, but also occasionally HPV-2, in up to 85% of these lesions (SYRJANEN et al. 1986; EVERSOLE et al. 1987).

The histology and the HPV types are thus similar both in oral papillomas and condylomas.

### 3.1.6 Common Wart (Verruca Vulgaris)

Occasional case reports have described common warts in the oral cavity usually as firm, whitish, sessile, circumscribed, exophytic lesions on the lips, with hyperkeratinisation of the superficial epithelia and elongation of the rete rides which, at the margins, usually bend inward toward the centre of the lesion. HPV-6, -11 and -16 and cutaneous HPV types, e.g. HPV-1, -2, -4 and -7, have been found in oral common warts in some studies (NAGASHEAR et al. 1985; ADLER-STORTHZ et al. 1986; ZEUSS et al. 1991; EVERSOLE et al. 1987). Indeed, HPV-2 and HPV-4 have been detected in more than 55% of oral warts (DE VILLIERS 1989), and HPV-57 DNA has also recently been demonstrated in the majority of oral verrucae (DE VILLIERS et al. 1989; PADAYACHEE 1994).

It is probably appropriate that the term oral wart should be restricted to lesions associated with the cutaneous HPV types while, if the mucosal HPV types (6 and 11) are found, the lesions are really oral condylomata or papillomas. This distinction has implications in tracing the source of HPV infection, i.e. oro-genital contact versus oro-cutaneous contact.

### 3.1.7 Focal Epithelial Hyperplasia

Focal epithelial hyperplasia, or Heck's disease, is a benign lesion of the oral mucosa originally thought to be restricted to certain ethnic groups, particularly Inuits and natural Indians from North and South America. These lesions, however, have subsequently been found, albeit rarely, in many other ethnic groups around the world.

Focal epithelial hyperplasia is a manifestation of HPV infection in the oral cavity in individuals with a specific genetic predisposition, as is the case in epidermodysplasia verruciformis lesions of the skin, described above. Papillomavirus particles and HPV antigens have been repeatedly observed in focal epithelial hyperplasia lesions, and it is now evident that more than 90% of biopsies from the hyperplasia contain HPV-13 and/or -32 DNA (BEAUDENON et al. 1987; GARLICK et al. 1989; HENKE et al. 1989).

### 3.1.8 Oral Warty Lesions

HPV-7, -13, -18 and -32 have been found in oral warty lesions in HIV infection (GREENSPAN et al. 1988). HPV may also be present in the oral mucosa in HIV

infection in the absence of clinical disease (SNIJDERS et al. 1990), as they may be in otherwise healthy persons.

### 3.1.9 Diagnosis of Human Papillomavirus-Induced Lesions

Until quite recently, HPV diagnosis was based exclusively on the morphological criteria seen on histopathology, because HPV cannot be cultivated and diagnostic serology has not been widely available. The morphological change particularly characteristic of HPV infection is koilocytosis, in which keratinocytes typically in the intermediate layer of the epithelium are hyperchromatic, with slightly irregular nuclei surrounded by a distinct cytoplasmic clear zone (halo). At the margins of the halo, the cytoplasm is condensed and usually exhibits an amphophilic-staining pattern. Koilocytic cells are still regarded as the most reliable morphological criteria for diagnosis of HPV infection (SYRJANEN, 1989). However, there can be difficulties in the distinction between true koilocytes and other vacuolised cells, and this is important, especially in the oral cavity, where different types of vacuolised cells unrelated to infection are not infrequently seen (KELLOWSKI et al. 1990). Another cytological change frequently associated with HPV infection is the presence of parakeratotic (dyskeratotic) cells either singly or in clusters. Both the koilocytotic and dyskeratotic keratinocytes frequently show bi- or multinucleation (SYRJANEN 1989).

Various HPV antigens can be detected immunocytochemically, but the most *reliable* method so far to diagnose HPV infection is to detect HPV nucleic acid in the lesion by hybridisation methods or gene amplification. These techniques have now largely replaced immunocytochemistry, and there are several commercial kits available for diagnosis in biopsies based on *in situ*, dot blot, or Southern blot hybridization methods. PCR is the only method which can also be used to reliably detect latent HPV infections in oral squames rather than biopsies (SYRJANEN 1990; JALAL et al., in press).

### 3.1.10 Management of Human Papillomavirus-Related Oral Lesions

Surgery is the usual management of HPV-related lesions, but retinoids may be effective (BURG and SOBETZKO 1990) and interferon may be used.

### 3.1.11 Relation to Other Oral Diseases

*3.1.11.1 Lichen Planus.* Oral lichen planus specimens may show positive immunostaining for HPV structural proteins (KASHIMA et al. 1990), and *in situ* hybridization has shown HPV-11, -16 and HPV-16-related virus (SYRJANEN et al. 1986; MAITLAND et al. 1987; COX et al. 1993); however, for the reasons discussed above, the presence of HPV in oral lichen planus lesions does not prove an aetiological link. Other investigators have not found HPV in lichen planus (YOUNG and MIN 1991).

*3.1.11.2 Oral Leukoplakia (Keratosis).* Though some have not found HPV DNA in keratoses (YOUNG and MIN, 1991), HPV-suggestive changes have been noted in some lesions and, subsequently, HPV antigens and DNA from HPV-2, -6, -11 and -16 have been found by several groups (SYRJANEN et al. 1986; GREER et al. 1990; KASHIMA et al. 1990; ABDELSAYD 1991). Indeed, we and others have shown that HPV-16 and HPV-16-related sequences can be detected in more than 80% of keratoses (KASHIMA et al. 1990; SYRJANEN et al. 1988; MAITLAND et al. 1989). Nevertheless, the presence of HPV in *normal* oral mucosa casts serious doubt on any regular causal relationship, though HPV-2 and -6 DNA have been found in smokeless tobacco-related keratoses (GREER et al. 1987, 1990), and HPV-16 is strongly associated with proliferative verrucous leukoplakia (PALEFSKY et al. 1995; SHROYER and GREER 1991).

Recent studies have discounted the presence of HPV in HL (SYRJANEN et al. 1989; FICARRA et al. 1988; ALESSI et al. 1990), and it now appears certain that HL is related to EBV (see p. 47).

*3.1.11.3 Verrucous Carcinoma.* Oral verrucous carcinoma is clearly aetiologically linked with tobacco consumption and betel chewing (CHANG et al. 1991). However, viral involvement in verrucous carcinomas has long been suspected at other body sites (e.g. larynx and the ano-genital area), and the morphological features in oral verrucous carcinoma of papillomatosis, dyskeratosis and koilocytosis support an HPV aetiology (CHANG et al. 1990). Despite this, early studies found no or only weak evidence for HPV in oral verrucous carcinoma (YOUNG and MIN, 1991; ADLER-STORTHZ et al. 1986; JOHNSON et al. 1991). However, recent work has demonstrated HPV-6, HPV-11 (SHROYER et al. 1993), HPV-18 (NOBLE-TOPHAM et al. 1993) and HPV-2 (CHANG et al. 1992) in oral verrucous carcinoma.

*3.1.11.4 Squamous Cell Carcinoma.* Oral squamous cell carcinoma is clearly aetiologically linked with tobacco and/or alcohol use. A substantial portion of these tumors, however, also contain HPV sequences, often of the "high-risk" group (Table 6). The rate of HPV detection varies in reports based on clinical material from oral squamous cell carcinoma from 0% to 94% (Table 6), but the ability to detect HPV is strongly dependent on the sensitivity of the method used, as well as the representativeness of the sample analysed, and is higher in most of the more recent studies (KASHIMA et al. 1990; WATTS et al. 1991; WOODS et al. 1993; COX et al. 1993; MILLER et al. 1994; OSTWALD et al. 1994). Oral squamous cell carcinoma biopsies, when analysed by *in situ* hybridisation and PCR, disclosed HPV-11, -16 or -18 DNA sequences in 10%–60% (CHANG et al. 1990; TSUCHIYA et al. 1991; SHINDOH et al. 1992; TYAN et al. 1993; WOODS et al. 1993; OSTWALD et al. 1994; MILLER et al. 1994). An HPV-16-related virus was found by us in about 40% (MAITLAND et al. 1989). HPV DNA sequences may also be found in leucocytes from patients with oral carcinoma (HONIG et al. 1995).

Most carcinoma-derived cell lines contain integrated HPV DNA genomes, the integrations causing disruptions and deletions within the early viral genes, whereas in benign lesions HPV DNA is present almost exclusively as free, monomeric or oligomeric episomes.

**Table 6.** Frequency of human papillomavirus (HPV) nucleic acid detection in oral squamous carcinomas

| Sample size | Frequency (%) | HPV types | DNA detection method | Reference |
|---|---|---|---|---|
| 7 | 43 | 1–19, 21–25 | Southern blot | De Villiers 1985 |
| 6 | 50 | 11, 16 | Southern blot | Loning et al. 1985 |
| 5 | 80 | 6, 11, 16, 18 | In situ | Dekmezian et al. 1987 |
| 13 | 39 | 6/11, 16/18 | Dot blot | Loning et al. 1987 |
| 51 | 12 | 6, 11, 13, 16, 18, 30 | In situ | Syrjanen et al. 1988 |
| 36 | 14 | 11, 16, 18 | Southern blot | Brandsma and Abramson 1989 |
| 7 | 28 | 16, 18 | PCR-dot blot | Kiyabu et al. 1989 |
| 15 | 46 | 16 | Southern blot | Maitland et al. 1987 |
| 17 | 77 | 6, 11, 16, 18 | Southern blot | Chang et al. 1989 |
| 8 | 50 | 16 | PCR | Maitland et al. 1989 |
| 40 | 6 | 6, 11, 16, 18 | In situ | Chang et al. 1990 |
| 40 | 27 | 6, 11, 16, 18 | PCR | Chang et al. 1990 |
| 50 | 6 | 6, 11, 16, 18, 31, 33, 35 | In situ | Greer et al. 1990 |
| 10 | 10 | 16 | In situ and PCR | Shroyer and Greer 1991 |
| 24 | 13 | 6/11, 16/18 | Southern blot | Tsuchiya et al. 1991 |
| 23 | 68 | 6/11, 16/18 | Southern blot | Watts et al. 1991 |
| 23 | 94 | 6, 11, 16, 18 | PCR | Watts et al. 1991 |
| 39 | 49 | 4, 16, 18 | PCR/Southern blot | Yeudall and Campo 1991 |
| 17 | 0 | 16/18, 31/33/35 | In situ | Young and Min 1991 |
| 17 | 18 | 16 | PCR/Southern blot | Brachman et al. 1992 |
| 118 | 25 | 6, 16 | PCR/Southern blot | Madden et al. 1992 |
| 24 | 33 | 16 | PCR | Shindoh et al. 1992 |
| 8 | 50 | 16 | Southern blot | Cox et al. 1993 |
| 30 | 14 | 16 | PCR/In situ | Frazer et al. 1993 |
| 9 | 11 | 16 | PCR | Tyan et al. 1993 |
| 18 | 77 | Various | PCR/Southern blot | Woods et al. 1993 |
| 27 | 22 | 16/18 | PCR/Southern blot | Anderson et al. 1994 |
| 64 | 25 | Various | PCR/Southern blot | Brandwein et al. 1994 |
| 30 | 67 | 16/18 | PCR | Miller et al. 1994 |
| 26 | 62 | 6/11, 16, 18 | PCR/Southern blot | Ostwald et al. 1994 |

PCR, polymerase chain reaction.

HPV can, with chemical carcinogens, transform oral keratinocytes (Shin et al. 1994). Interestingly, only the HPV types closely associated with malignancies have immortalising activities; the benign HPV types are unable to function in this way (Woodworth et al. 1989). Transformation studies with oncogenic HPV DNA has localised the transforming activity mainly to the E6 and E7 genes. However, despite the central role of these early HPV genes, malignant transformation appears to require additional factors such as co-infection with HSV or HCMV, exposure to tobacco alcohol, glucocorticoids or other hormones, or possibly other co-factors (Syrjanen and Scully, in press; Woodworth et al. 1989; Mitrani-Rosenbaum et al. 1989; Pater et al. 1990).

Although the E7 protein alone has transforming and immortalising activities in rodent cells, cooperation between E6 and E7 appears to be both necessary and

sufficient for the efficient immortalisation of primary human genital keratinocytes. Sequence rearrangements in URR may also be responsible for the oncogenicity of specific HPV types (KITASATO et al. 1994). The E5 gene may also be involved, since the E5 protein alters responses of the cell receptor tyrosine kinases (MARTIN et al. 1989) and can thus modulate epidermal growth factor receptor (EGFR) activity. Protein (oncoproteins) encoded by the E6 and E7 genes from "high cancer risk" HPV types are able to interfere with some cellular growth-regulatory proteins (WERNESS et al. 1990; MUNGER et al. 1989). The E6 oncoprotein can bind to and interfere with the p53 tumour suppressor gene product (WERNESS et al. 1990). The E7 oncoprotein can bind to and interfere with the retinoblastoma tumour suppressor gene product pRB (MUNGER et al. 1989). The importance of the E6–p53 and E7–pRB interactions is substantiated by the observation that there is a correlation between the ability of the HPV genes to bind these cell growth-regulatory proteins and the oncogenicity of the HPV type. High risk-type viruses HPV-16 and HPV-18 E6 proteins can associate with p53, whereas no complex can be detected with HPV-6 or HPV-11 ("low-risk") E6 proteins (WERNESS et al. 1990). E7 proteins from the oncogenic HPV types of 16 and 18 bind pRB more strongly than does E7 from the benign HPV types 6 and 11 (MUNGER et al. 1989). Only a few studies exist on HPV E6 or E7 gene expression in carcinomas originating from head or neck region, but tonsillar carcinomas express high levels of HPV-16/E6/E7 transcripts which originate from integrated as well as episomal HPV DNA (SNIJDERS et al. 1992).

These interactions provide at least a theoretical model as to how HPV might be involved in carcinogenesis. Normal p53 seems to act as a tumour suppressor because it functions as a guardian of the genome, thus maintaining its integrity. Damage to cellular DNA leads to accumulation of p53 and to cessation of cell proliferation, providing time for the cell DNA repair mechanisms to act. If the repair fails, p53 can trigger cell "suicide" through apoptosis. Those cells that carry a mutation in p53 or carry an oncogenic virus may become genetically unstable, which can lead to other mutations and chromosome aberrations and finally to selection of malignant cell clones. Interestingly, we and others have demonstrated p53 mutations in oral carcinomas (SOMERS et al. 1992; SAKAI and TSUCHIDA 1992; GUSTERSON et al. 1991; FIELD et al. 1991, 1992; WARNAKULASURIYA and JOHNSON 1992; OGDEN et al. 1992; MATTHEWS et al. 1993). It has been shown that *cervical* carcinoma cell lines which are HPV negative have a mutation in either p53 or RB gene; HPV-positive carcinomas have been shown to express wild-type p53 and pRB (MUNGER et al. 1989; SCHEFFNER et al. 1990). The hypothesis is that the inactivation of normal function of p53 (SCHEFFNER et al. 1990) or pRB (or the related p107) proteins is a critical step in squamous cell carcinogenesis.

H-*ras* oncogene mutation also appears correlated with HPV in oral carcinoma (ANDERSON et al. 1994), and it is clear that some HPV can interact with various transcription factors, especially NF-1 (nuclear factor-1), API (includes oncogenes *jun* and *fos*) (BERNARD and APT, 1994), protein phosphatase 2A (PP2A) and others (ZUR HAUSEN and DE VILLIERS 1994).

Recently, the possible aetiological role of HPV infection in the pathogenesis of oral pre-cancer lesions and cancer has been supported by the discovery of HPV-suggestive lesions in oral pre-cancer specimens as well as by DNA hybridisation

studies disclosing HPV-11, -16 and -18 DNA (CHANG et al. 1991; SCULLY et al. 1988; CHANG et al. 1990; SYRJANEN et al. 1988; OSTWALD et al. 1994).

*3.1.11.5 Spindle Cell Carcinoma.* Interestingly, spindle cell oral carcinomas appear not to contain HPV (LARSEN et al. 1994).

*3.1.11.6 Nasopharyngeal Carcinoma.* HPV-11 and HPV-16 may be detected in nasopharyngeal carcinoma (TYAN et al. 1993; HORDING et al. 1994).

### 3.1.12 Conclusion

HPV are present in oral squamous cell carcinomas and fulfil most of the criteria needed for oncogenicity, but their role in oral cancer still needs further elaboration. HPV may be implicated in oral carcinogenesis, though the evidence is as yet less convincing than it is for other mucosal carcinomas (YEUDALL 1992). Newer techniques of nucleic acid technology will undoubtedly reveal a clearer picture of this rapidly expanding field of research.

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# Oral Pathology of Acquired Immunodeficiency Syndrome and Oro-facial Kaposi's Sarcoma*

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*This work is dedicated to the late Professor Jens J. Pindborg, Copenhagen.

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## 1 Introduction

The present volume on oral pathology focuses on diagnostic and prognostic aspects of oral diseases. The acquired immunodeficiency syndrome (AIDS) was first described in 1981, and diagnostic criteria for both the clinical and histopathological aspects of the disease were considered to be of particular importance. Early adequate diagnosis of oral lesions, which in many cases are indicators of the human immunodeficiency virus (HIV) disease, is relevant for early treatment and thus to a certain extent prognosis.

### 1.1 Factors Relevant to Diagnosis

Clinical and histopathological diagnosis of oral lesions associated with HIV infection and AIDS may be influenced by several parameters. Whether or not the HIV serostatus is known may play a certain role in the diagnosis. In cases in which the HIV-positive serostatus is known, diagnoses such as hairy leukoplakia (HL) and Kaposi's sarcoma (KS) may be biased by this knowledge. In addition, the stage of the disease – according to the Centers for Disease Control (CDC) classification – may also influence diagnostic decision processes. A number of pitfalls have been described both for clinical and histopathological diagnoses. Exact knowledge of the history and serostatus of a patient will enable the oral pathologist to arrive at an adequate diagnosis. Close cooperation between the clinician and the oral pathologist is warranted.

### 1.2 Prognosis

Some oral manifestations have been recognised as markers and indicators of HIV infection and AIDS. Oral candidiasis, HL and KS, in particular, need to be mentioned as markers. The prognostic value of HL for the development and course of the disease has been clearly shown. Some other opportunistic infections such as herpes zoster and cytomegalovirus (CMV) infection have some prognostic significance for the development and course of HIV disease. The prognosis for patients with HIV infection has been improved mainly through improved control of opportunistic infections such as candidiasis, toxoplasmosis, *Pneumocystis carinii* pneumonia infection (PcP) and viral diseases.

## 2 Classification

Several classifications of oral manifestations in HIV infection have been published and revised. The EC Clearinghouse on Oral Problems Related to HIV Infection and WHO Collaborating Centre on Oral Manifestations of the Immunodeficiency

Virus published a classification and diagnostic criteria for oral lesions in HIV infection in 1993 (EC 1993). The classification was based on presumptive and definitive criteria.

### 2.1 Presumptive and Definite Criteria for the Diagnosis of Oral Lesions in Human Immunodeficiency Virus Infection

Presumptive criteria predominantly relate to the initial clinical appearance of a lesion. These diagnostic criteria were not considered to be perfect because of the fact that patients with other diseases may present with similar appearances. A working knowledge of oral mucosal diseases is mandatory, and the spectrum of differential diagnoses must always be considered. The definite criteria are those necessary to establish a reliable diagnosis requiring further clinical or laboratory tests. These include histopathological procedures and the application of refined techniques such as electron microscopy, in situ hybridisation, polymerase chain reaction (PCR) and other molecular biological techniques.

### 2.2 Classification Groups

Oral lesions in HIV infection were classified into three groups:

- *Group I*: Lesions strongly associated with HIV infection, including candidiasis, HL, several types of periodontal disease, KS and non-Hodgkin's lymphoma.
- *Group II*: Lesions less commonly associated with HIV infection, including some bacterial infections, salivary gland disease, ulcerations not otherwise specified (NOS) and a number of viral infections both of the herpes virus group and the human papillomavirus group.
- *GroupIII*: Lesions seen in HIV infection, some of which are rare and therefore may only have been recorded anecdotally. Bacterial, fungal and viral infections as well as drug reactions or neurological disturbances have been recorded.

Since HIV infection has become a pandemic, geographical differences of manifestations may still be noticed in the future. Tropical diseases such as mycotic infections or leprosy and numerous other diseases may make further revisions of the present classification necessary.

## 3 Lesions Strongly Associated with Human Immunodeficiency Virus Infection (Group I)

### 3.1 Candidiasis

Oral candidiasis, the "disease of the diseased", is probably one of the most frequent opportunistic infections in the course of HIV infection. During HIV infection, all

patients develop oral candidiasis at some time of the disease process. It has been considered as a forerunner of AIDS, and an average of one in three patients are affected by some type of oral candidiasis (Samaranayake 1992; Samaranayake and Holmstrup 1989). Of particular importance is the fact that oral candidiasis is not usually observed in individuals 25–54 years of age, an age-group typically affected by HIV infection. Occurrence of signs of oral candidiasis in this group of patients suggests an underlying immuno-incompetence, probably related to an HIV infection. From the clinical point of view, the appearance of the variants of oral candidiasis is similar in the immunocompetent and immunocompromised subjects. Some variations, however, exist in the clinical pattern of oral candidiasis associated with HIV infection, and these are related to factors such as inadequate dosage of antifungals, poor compliance, shift of species or resistance to drugs. All areas of the oral cavity may be affected by oral candidiasis, however, the buccal mucosa, the tongue, the palate and the floor of the mouth are involved most frequently. Different types of oral candidiasis may be seen in one patient.

### 3.1.1 Erythematous Candidiasis

Erythematous candidiasis may be easily overlooked, particularly in patients whose HIV serostatus is unknown. Diagnostic presumptive criteria are red areas usually located on the palate and dorsum of the tongue, but occasionally on the buccal mucosa. White spots and plaques may be seen, but these are not usually conspicuous. Figure 1a shows a characteristic erythematous candidiasis of the dorsum of tongue characterised by loss of papillae with a glossy red appearance. Clinical symptoms are minimal. Due to contact of the tongue with the palate, a palatal erythematous candidiasis is usually observed at the same time. The undefined nature of erythematous candidiasis is exemplified by the relatively poor definitive criteria. The detection of *Candida albicans* and/or response to antifungal therapy may help to establish the diagnosis.

*3.1.1.1 Laboratory Findings.* As mentioned above, the detection of *Candida albicans* by microbiological culture may help to establish the diagnosis. It must, however, be remembered that *Candida albicans* is a saprophyte of the oral cavity and as such may be found in up to 20%–50% of normal individuals. Quantification of *Candida albicans* may be helpful, and it has been shown that the number of colony-forming units of *Candida albicans* may be considered as an indicator for the presence of clinical disease. Smears to demonstrate blastospores or candidal hyphae may be helpful, although the latter are not usually found in erythematous candidiasis. The fact that *Candida albicans* is a dimorphic agent causing different types of oral candidiasis further complicates the situation.

*3.1.1.2 Histopathology.* No systematic studies of the histopathology of erythematous candidiasis associated with HIV infection exist.

Histopathologically, erythematous candidiasis is characterised by an epithelial atrophy with loss of rete ridges, subepithelial inflammatory infiltration,

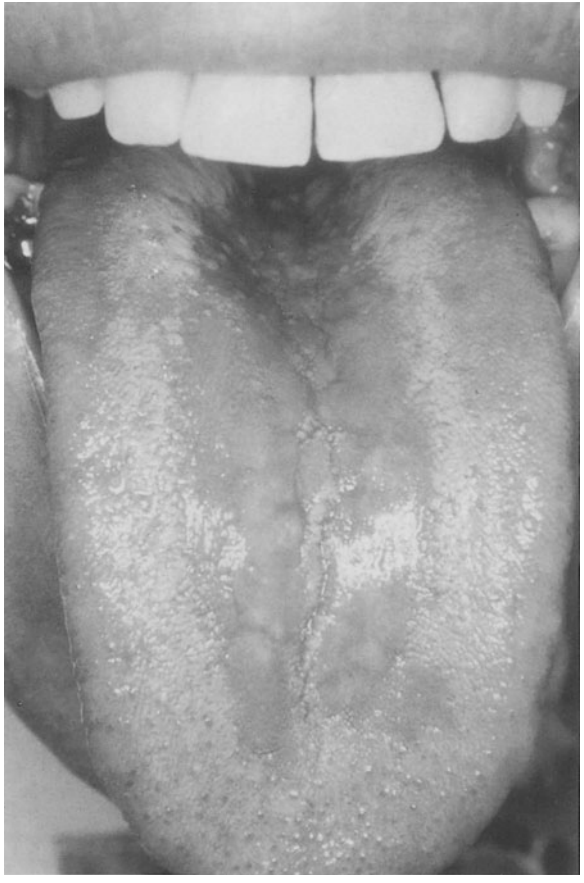
hyperaemia and occasional microabscesses. Penetration of the epithelium with hyphae is the exception. Preliminary studies have been conducted to show the significance of calprotectin, an antibacterial and antimycotic protein located in keratinocytes of normal mucosa and that of erythematous and other types of candidiasis (EVERSOLE et al. 1993). It was demonstrated that calprotectin production in oral epithelium of erythematous and pseudomembranous candidiasis is up-regulated (Fig. 1b).

### 3.1.2 Pseudomembranous Candidiasis

Presumptive criteria of pseudomembranous candidiasis are white or yellow spots or plaques that may be located in any part of the oral cavity and can be wiped off to reveal an erythematous surface which may bleed. The definite criteria for the demonstration of pseudomembranous candidiasis are response of the lesions to antifungal therapy and tests for the presence of *Candida albicans*. These are not essential for diagnosis, but they may enhance it, particularly in cases resistant to antifungal therapy. Smears and/or cultures may be performed (EC 1993). Cytological smears, however, stained by periodic acid–Schiff (PAS) are revealing when the presence of candidal hyphae is shown.

*3.1.2.1 Histopathology.* As with erythematous candidiasis, no systematic studies on the histopathology of pseudomembranous candidiasis in HIV infection have been published. The indicative finding is the demonstration of hyphae in the surface epithelium down to the spinous cell layer. Parakeratosis, acanthosis and spongiosis may be seen. Intra-epithelial microabscesses are characteristic. Subepithelially, some inflammatory changes may be observed, although these may be minimal due to the immunodeficiency and lack of response. Figure 1c shows the hyphae in a biopsy of pseudomembranous candidiasis. Occasionally, deeper penetration of hyphae beyond the basal membrane into the subepithelial connective tissue has been observed (REICHART et al. 1995). In an ultrastructural study of pseudomembranous candidiasis, the principle of thigmotropism of *Candida albicans* was shown (REICHART et al. 1995). In addition, ability of the hyphae to detach desmosomes has been demonstrated (Fig. 1d,e).

**Fig. 1.** **a** Erythematous candidiasis of the dorsum of tongue in a human immunodeficiency virus (HIV)-positive patient. The red, glossy appearance is characteristic. **b** Section of pseudomembranous candidiasis stained with antibodies against calprotectin showing marked staining of the superficial epithelial cells. Moderate inflammatory infiltration is seen subepithelially. APAAP, ×250. **c** Same patient as in Fig. 1b, revealing the characteristic hyphae in the superficial layer of the epithelium. PAS, ×250. **d** Transmission electron microscopy of pseudomembranous candidiasis. Two cross-sections of hyphae, one in the intercellular space, the other through an epithelial cell, are seen. In addition, multiple bacteria are also found. ×30000. **e** Scanning electron microscopical view of epithelial surface of a pseudomembranous candidiasis. Hyphae are seen penetrating the surface in several locations. The penetration in between epithelial gaps is called "thigmotropism". ×3000



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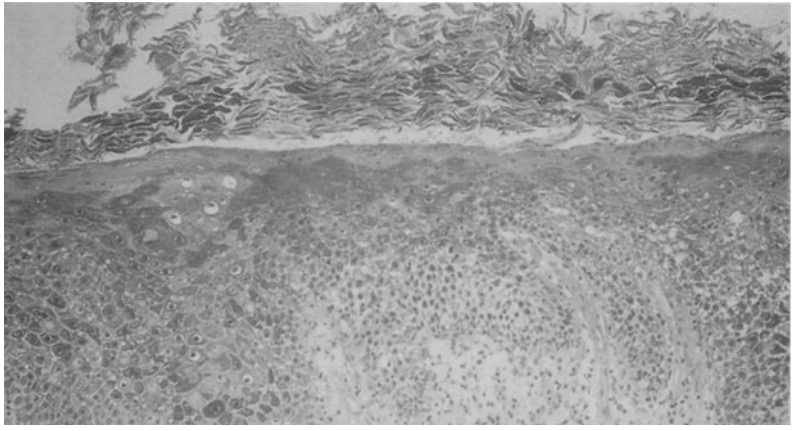

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### 3.1.3 Other Types of Oral Candidiasis

Angular cheilitis and denture-induced stomatitis may be observed in patients with HIV infection. Both variants of oral candidiasis can be diagnosed clinically. Recently, a papillary variant of oral candidiasis in HIV-infected patients has been described (REICHART et al. 1994).

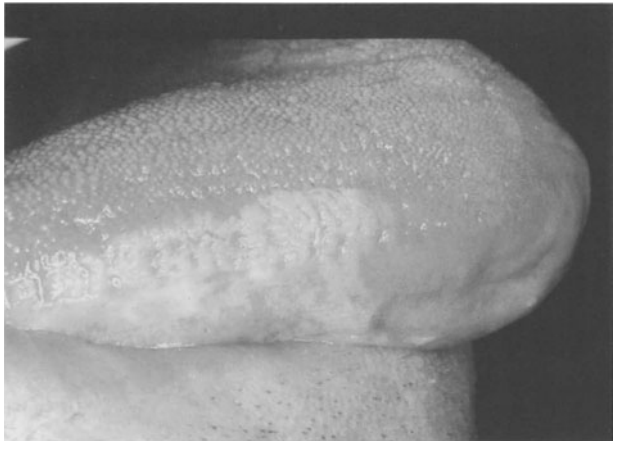
### 3.1.4 Prognosis

The prognosis of oral candidiasis in HIV infection is determined by the fact that eradication of *Candida albicans* by antifungal treatment is not possible. Antifungal medication results in disappearance of clinical signs and symptoms; however, recolonisation occurs as soon as treatment is interrupted or dosage is reduced. Dentinal caries has recently been described as a reservoir for *Candida albicans* (ROEDER et al. 1995). In some centres, prophylaxis has been introduced in patients whose $CD4^+$ cell count is below $200/mm^3$. Azoles, particularly fluconazole and itraconazole, are the drugs of choice, but a number of patients in the final stage of AIDS develop resistance to azole therapy.

## 3.2 Hairy Leukoplakia

HL, an Epstein-Barr virus (EBV)-associated lesion, has been described in both immunodeficient and immunocompetent individuals (for a review, see PINDBORG and REICHART 1995). It has been observed in all risk groups and occurs worldwide. It has not been found in any other mucosal lining except the lateral border of the tongue and, very rarely, the buccal mucosa. There is no explanation of why HL preferentially occurs in this site. For correct diagnosis, both clinical and laboratory findings are necessary. Clinical presumptive criteria are bilateral whitish/grey lesions on the lateral margins of the tongue. Vertical corrugations are typical, and the lesion can not be removed. HL may extend to the ventral and dorsal surfaces of the tongue, where it is usually flat. The most reliable definitive criterion is the demonstration of EBV in the lesions. Persistence of the white lesions after antifungal treatment supports the diagnosis of HL. Histological features are not sufficiently specific to be acceptable as definitive criteria. Figure 2a shows an example of HL at the lateral border of tongue in an HIV-infected patient.

**Fig. 2.** **a** Hairy leukoplakia at the lateral border of tongue revealing the corrugated pattern partially extending to the lower surface of the tongue. **b** Biopsy of hairy leukoplakia showing surface epithelium and intense staining for virus capsid antigen of numerous nuclei, revealing the presence of Epstein-Barr virus. APAAP, ×200. **c** Transmission electron micrograph of a thin section of hairy leukoplakia, revealing multiple herpesvirus-like particles in the interepithelial space. ×25 000



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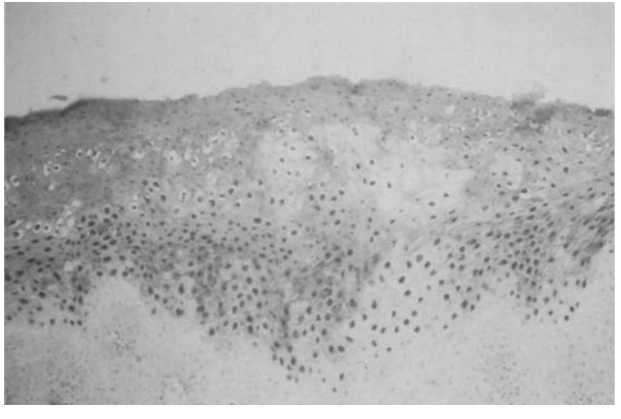

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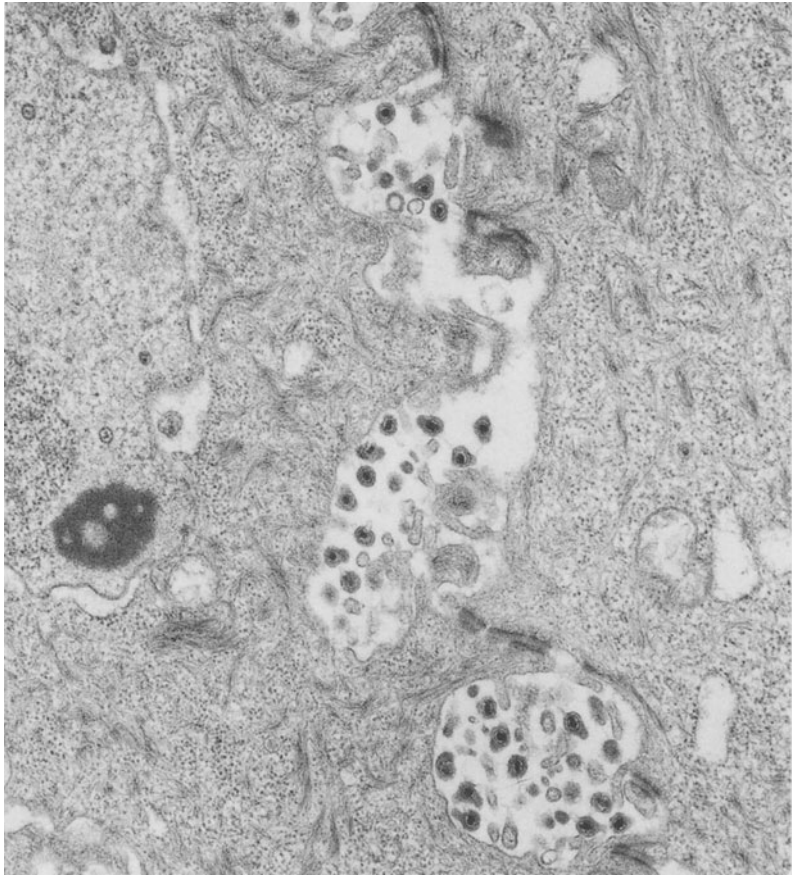

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### 3.2.1 Histopathology

When HL was first described in 1984, it was compared to the histopathology of the flat wart of the skin. Histological features characterising HL are keratin projections, parakeratosis and acanthosis, ballooning of cells in the prickle cell layer, little or no inflammation and mild epithelial atypia in some cases. Basophilic nuclear inclusion bodies of epithelial cells in the prickle cell layer are an indication of viral infection. In 43%–100% of cases of HL, candidal infection may be demonstrated in PAS sections. An absence of Langerhans cells in HL has been described (DANIELS et al. 1987). Due to the unspecified histological appearance of HL, but also due to problems related to sectioning (ANDERSEN et al. 1990) and bias caused by lack of information about the HIV serostatus of the patient, HL may become a pitfall from the morphological point of view (SCHULTEN et al. 1991). To avoid misinterpretations, demonstration of EBV is mandatory. Numerous techniques have been suggested to demonstrate EBV, including immunohistochemistry (Fig. 2b), in situ hybridisation, negative-staining electron microscopy and electron microscopy (EPSTEIN et al. 1995). The disadvantage of electron microscopy is that this method allows for the demonstration of herpesvirus-type particles but not for the identification of EBV. Demonstration of viral capsid antigen in either biopsies or cytological smears allows for the unequivocal demonstration of EBV. A number of electron microscopy studies on the fine structure of HL have been published (ZHANG et al. 1988; GREENSPAN et al. 1989; EL-LABBAN et al. 1990). The presence of *Candida albicans* hyphae was revealed electron microscopically in a number of cases. Characteristic koilocyte-like cells showed pycnotic nuclei and condensed chromatin. Virus particles of the herpesvirus group may be observed in and around koilocytotic cells as well as in intercellular spaces. Isometric viral nucleocapsids have a diameter of 100 nm. Budding processes may also be observed. Fully assembled herpes-virus-type particles have an identical diameter of 150 nm (Fig. 2c). In addition, tubulo-reticular structures with a diameter of 35 nm were observed, and remnants of membrane were found in close approximation to the nuclei of koilocytotic cells. One study has shown that the expression of proteins encoded by EBV-transactivated genes depends on the differentiation of epithelial cells in HL (BECKER et al. 1991a). The EBV immediate early gene product BZLF1 was localised to the cytoplasm of the basal epithelial layer by indirect immunofluorescence. To date, this is the only study in which it was possible to demonstrate that, in immunocompromised and probably also in immunocompetent patients, EBV may move with increasing differentiation from the cytoplasm to the nucleus of epithelial cells, where it is co-activated during the terminal differentiation of epithelium at the lateral border of the tongue. Figure 2c shows herpesvirus-type viral particles in a biopsy of HL which were shown to be EBV by immunohistochemical methods. Non-invasive methods to prove the presence of EBV in lesions which clinically seem to be HL are easier to perform, because it may be difficult to take a biopsy due to lack of complicance of many HIV-infected patients.

### 3.2.2 Prognosis

Although HL is associated with EBV, this lesion has not been demonstrated to be pre-cancerous. Single cases of oral carcinoma, however, have been described. The presence of HL is considered as a predictor for the development of AIDS, and three quarters of patients with HL develop AIDS in 2–3 years (MONIACI et al. 1990). Treatment of HL is unnecessary in most cases, but regression of the lesion has been observed concurrent with antiviral treatment for other viral infections. HL lesions seem to recur after discontinuation of treatment.

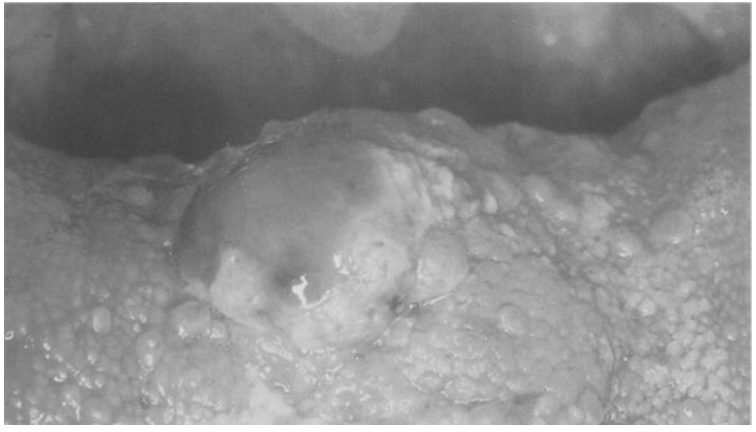
## 3.3 Kaposi's Sarcoma

In 1872, M. Kaposi first described a lesion which he termed idiopathic multiple pigmented sarcoma of skin. This type of classical KS was rare and mainly occurred in elderly men of southern and eastern European descent. Oral manifestations of Kaposi's classical type were extremely rare. Four different subtypes of KS are currently differentiated:

1. The classical European or Mediterranean KS
2. The endemic African KS
3. KS occurring in transplant patients under immunosuppressive therapy
4. Epidemic KS associated to AIDS (eKS)

The fourth subtype, eKS, was first described in 1981 in young homosexual or bisexual men from New York. KS in HIV infection is an AIDS-defining lesion. Oral eKS was shown to predominantly occur in male homosexual patients (REICHART et al. 1993); it is rare in heterosexuals, intravenous drug users (IDU), women and children. Patients are affected at an average age of 38 years. Presumptive criteria describing and defining eKS are one or more erythematous, slightly bluish or violaceous macules or swellings with or without ulceration, predominantly seen on the palate, gingiva or tongue. (Fig. 3a). The characteristic histological appearance on biopsy allows a definitive diagnosis to be made. Most eKS lesions are 1–3 cm in diameter. Oral eKS lesions may be non-pigmented (REICHART and SCHIÖT 1989) and may also involve the jaw bones (LANGFORD et al. 1991a). Whenever the HIV serostatus is known, diagnosis of eKS is not to difficult. This is particularly so in those cases in which skin lesions precede those of the oral mucosa. Clinical

**Fig. 3.** **a** Oral Kaposi's sarcoma on dorsum of tongue, a characteristic site after palatal and gingival location. **b** Biopsy of Kaposi's sarcoma showing spindle-shaped cells and early formation of vessel-like structures. Numerous extravasated erythrocytes are also seen. H&E, ×200. **c** Semi-thin section of oral Kaposi's sarcoma. Large vascular spaces with protruding endothelial cells are seen. Toludine blue, ×350. **d** Transmission electron micrograph (TEM) of a thin section of oral Kaposi's sarcoma showing protruding endothelial cells in an newly formed vascular structure. Nuclei are irregular and large. ×5000. **e** TEM of tumour cells of Kaposi's sarcoma, which are often spindle-shaped with large oval nuclei with small rims of condensed chromatin. ×5000



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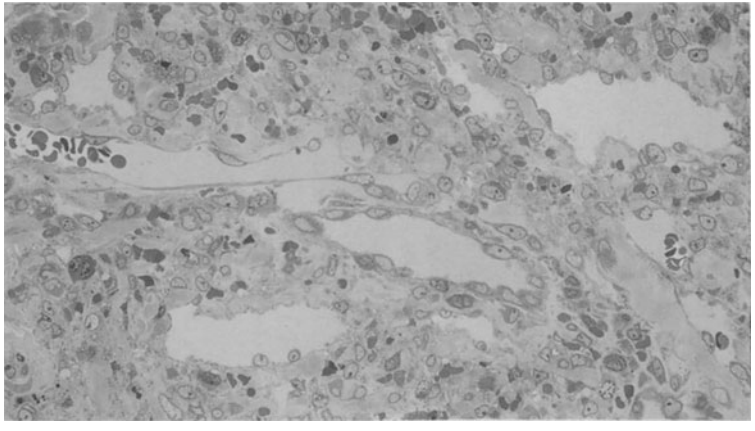

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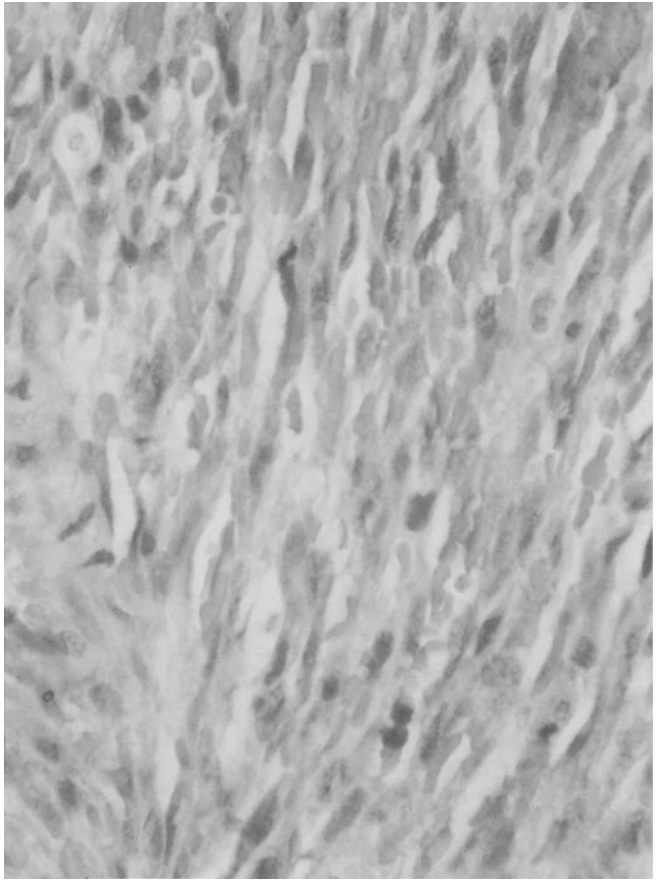

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differential diagnosis includes other pigmented lesions such as haematoma, haemangioma, tattoos and other neoplasms of vascular origin.

### 3.3.1 Histopathology

Light and electron microscopic features of the classical and African subtypes of KS and eKS are identical (GREEN et al. 1984). Since oral KS was extremely rare in the pre-AIDS era, this histopathological diagnosis was not routine. It has now become much more frequent, and oral pathologists must be aware of the fact that, histologically, eKS tissue may mimic granulation tissue with numerous vessels, as seen in peri-apical granuloma. Other lesions derived from vascular structures may also show similarities. Early eKS lesions show dilated, thin-walled vascular spaces with polymorphous endothelial cells. These protrude into the vascular lumen. Endothelial cells appear to be enlarged and show small amounts of cytoplasm containing polymorphic electron-lucent nuclei. Late stages of eKS characterised by tumour growth show a decrease in atypical vessels. The interstitial, spindle-shaped cells with enlarged oval nuclei prevail. Vessel-like spaces are filled with erythrocytes, and extravascular erythrocytes are common. Macrophages that phagocytose erythrocytes are seen, as are deposits of haemosiderin, and inflammatory cells may be seen around vessel-like spaces. Mitoses of tumour cells are rare (Fig. 3b,c). A 10-year retrospective, histopathological study of oral KS (REGEZI et al. 1993) has recently confirmed these findings. In addition, it was stated that eKS expresses the CD34 antigen, which may be useful in the diagnosis of small, well-delineated oral lesions lacking the classic KS features. Antigen CD34 may also be helpful to differentiate eKS from bacillary angiomatosis, pyogenic granuloma, epithelioid haemangioma or other spindle cell proliferations.

The ultrastructure of eKS shows a number of specific features. Endothelial cells are characterised by enlarged nuclei showing a marked lobulated surface and little marginal chromatin (Fig. 3d,e). Basal membranes are interrupted and connected by tight, intermediate or gap junctions or desmosomes. Weibel-Palade bodies are characteristic organelles of endothelial cells and are found in oral eKS lesions. In addition, intracytoplasmic aggregates of tubular structures with an average diameter of 24–35 $\mu$m are characteristic. Extravasated erythrocytes are seen on electron microscopy and in histopathology (KUNTZ et al. 1987). It was interesting to see similar ultrastructural findings in vessels of uninvolved oral mucosa of patients with HIV infection. Features as seen in eKS such as swollen endothelial cells, loss of cellular junctions, interruptions of the basal membrane, presence of Weibel-Palade bodies and tubular structures were also observed. These findings were interpreted as features of a disregulated vascular neogenesis (ZHANG et al. 1989). Studies of the extracellular matrix in eKS showed that stains for basement membrane components revealed distinct patterns of distribution. A delicate and partly fragmented lining of basement membrane around the sinusoid-like vascular spaces was seen, as well as an occasional diffuse interstitial fluorescence in the tumour stroma. From these findings, a vascular cell origin of the endothelial and spindle cell components of eKS was likely (BECKER et al. 1991b). Intense fluorescence was noted for procollagens type I and III and collagen

type VI. It was concluded that procollagens type I and III and collagen type VI are synthesized de novo by cells of the tumour stroma. In another study, immunoelectron microscopy showed an atypical pattern and a quantitive shift of collagens type I, III and VI in eKS. Collagen fibrils in eKS consisted of collagen type I. However, there was a marked loss of thick fibre bundles of collagens types I and III in eKS compared to normal oral mucosa. Collagen type VI was increased. This abundance of collagen type VI in a pattern comparable to early stages of wound healing suggested that the eKS stroma resembles an early organisational stage of the interstitial and vascular extracellular matrix. It appeared that the eKS stroma resulted from a continous autocrine and paracrine stimulation of cell growth and collagen synthesis (Becker et al. 1991c).

Other studies have focused on immunocompetent cells in eKS. Of interest was the fact that both eKS lesions and uninvolved oral mucosa of HIV-infected patients show infiltration with $CD4^+$ cells. In early eKS lesions, a marked increase of $CD8^+$ cells was found compared to later tumour stages. In addition, the number of HLA $DR^+$ cells was increased. Findings were interpreted as indicating the influence of eKS growth factors on the inflammatory reaction during the course of systemic immunosuppression characteristic of HIV infection (Tabata et al. 1993).

### 3.3.2 Aetiology and Pathogenesis

Both the aetiology and the pathogenesis of eKS are still unclear. However, as early as 1983 (Lozada et al. 1983) a number of possible factors which may be associated with eKS and probably other neoplasms were pinpointed, including higher than normal incidence of antibodies titers to CMV, multiple opportunistic infections (e.g. venereal diseases, herpetic infections, PcP, oral candidiasis), heavy drug usage and marked sexual activity. While some of these factors are still considered valid, the aetiologic relationship of HIV and eKS has also been discussed. There are several possible mechanisms by which oncogenic RNA viruses such as retroviruses may cause malignancy (Reichart 1991). In particular, the role of the HIV *tat* gene has been considered of importance (Cremer et al. 1990). Human papillomavirus type 16-related DNA sequences were found in eKS, and it was suggested that this may have a role in the pathogenesis of eKS (Huang et al. 1992). A transmissible agent was suspected, and eKS was considered a sexually transmitted infection (Beral et al. 1990). As such, eKS occurred in 1% of men with haemophilia and 21% of homosexual or bisexual men. While agents such as CMV, hepatitis B virus, human herpesvirus-6 (HHV-6), HIV and *Mycoplasma penetrans* have been suspected to be associated with eKS, in December 1994 herpesvirus-like DNA sequences in AIDS-associated KS were identified (Chang et al. 1994). These sequences were termed KS-associated herpesvirus-like sequences (KSHV), defining a possible new human herpesvirus. Of interest is the fact that these KSHV sequences are also found in HIV-negative patients with KS. In addition, KSHV DNA sequences are also found in AIDS-related body cavity-based lymphomas. The possible aetio-pathogenic role of KSHV is underlined by the observation that remissions of eKS were observed in patients who received foscarnet. Foscarnet is an antiviral drug used for treatment of infections with herpesviruses. Generally,

numerous growth factors have been identified which promote the growth of eKS cells. Autocrine and paracrine mechanisms are involved. A major growth factor for eKS has been identified as oncostatin M (NAIR et al. 1992).

Figure 4 shows a model for the pathogenesis of eKS. In principal, HIV-infected cells release viral and other cellular factors probably capable of stimulating activation and proliferation of cells of mesenchymal origin which may be endothelial cells. These acquire the spindle-shaped morphology characteristic of eKS cells. KS cells then start to produce cytokines, which maintain and amplify the cellular response via autocrine and paracrine pathways. Paracrine activation of normal cells leads to fibroblast proliferation, neoangiogenesis and inflammatory cell infiltration. If the initial stimulus persists, a vicious cycle may be established, resulting in tumour transformation.

It must be remembered that eKS is, by definition, a multicentric, hyperplastic proliferation and not a true neoplastic and metastatic expansion. As such, it is not a true sarcoma, at least at the beginning of the disease, but is considered as a type of multicentric hyperplasia.

### 3.3.3 Prognosis

The prognosis of oral eKS is poor. In one study (REICHART et al. 1993), average survival time of 124 patients was 1 year and 9 months (range, 3 months to 4 years and 6 months). A total of 57.3% of patients evaluated in that study died. Treatment is palliative, consisting of local or systemic administration of cytostatic drugs (bleomycin, vinblastine, vincristine, actinomycin D, doxorubicin, etoposide and others and combinations thereof). In addition, interferon-$\alpha_2$ has been used as an immunomodulatory treatment, and radiation (dosage, 20 Gy) has also been used. Treatment modalities and results for oral eKS are still not satisfactory, and it should be made clear that "many patients require infinite treatment for control of lesions."

## 3.4 Non-Hodgkin's Lymphoma

Non-Hodgkin's lymphoma (NHL) is observed in 77% of AIDS patients and Hodgkin's lymphoma in 23%. After eKS, HIV-related malignant lymphomas are the most common malignancies observed in AIDS patients. Presumptive criteria of NHL include firm, elastic, often reddish or purplish swellings with or without ulceration. The gingiva, palatal mucosa and fauces are the most common sites. Definite criteria are based on characteristic histological appearance on biopsy, supported by appropriate immunocytochemical or molecular biological investigations. Malignant lymphomas grow rapidly, and ulceration is therefore a common finding (LANGFORD et al. 1991b). When situated on gingival tissue, NHL lesions cannot be differentiated from necrotising stomatitis or infection. The histological spectrum of NHL is wide, including lymphoblastic, centroblastic, immunoblastic, highly malignant and unclassifiable types. Furthermore, the histological pattern of

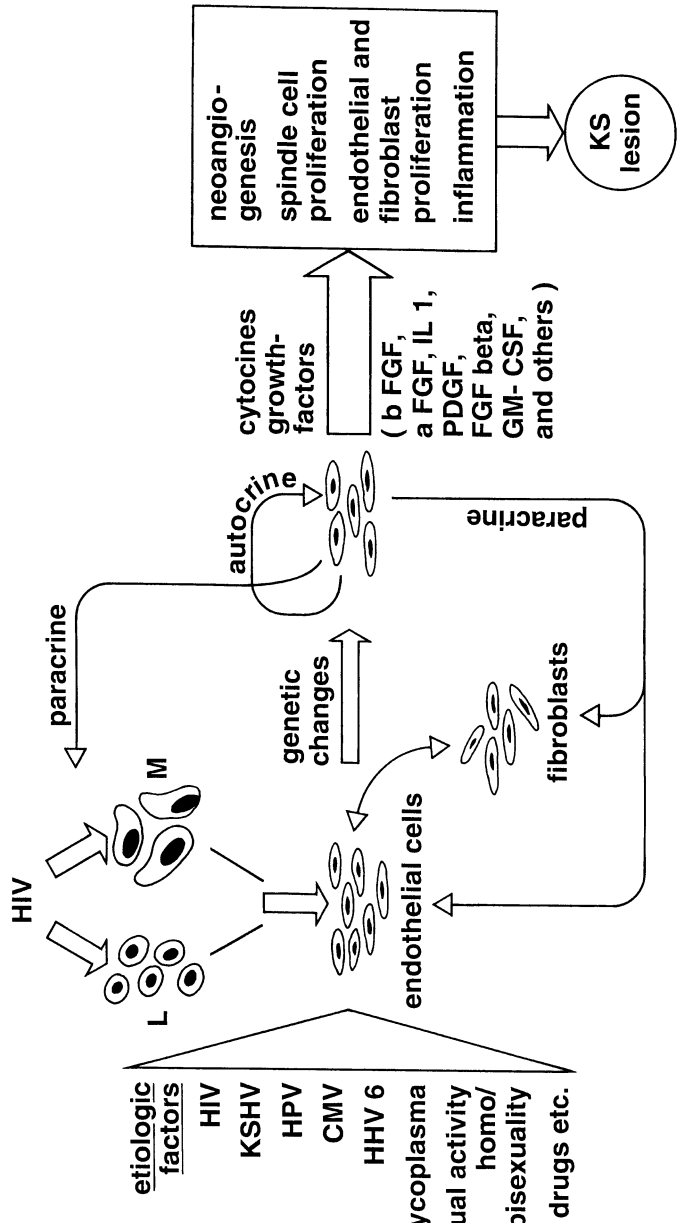



**Fig. 4.** Causative factors and pathogenesis of Kaposi's sarcoma (*KS*). Mainly autocrine and paracrine stimuli account for the formation of the KS tumour cell. *M*, macrophages; *L*, lymphocytes; *HIV*, human immunodeficiency virus; *KSHV*, KS herpesvirus; *HPV*, human papillomavirus; *CMV*, cytomegalovirus; *HHV*, human herpesvirus; *FGF*, fibroblast growth factor; *bFGF*, basic FGF; *aFGF*, acidic FGF; *PDGF*, platelet-derived growth factor; *GM-CSF*, granulocyte-macrophage colony-stimulating factor

non-Hodgkin's lymphoma ranges from large pleomorphic lymphoblasts to small, non-cleaved cells. A relation to EBV has been documented for both NHL and Hodgkin's lymphoma; however, not all B cell lymphomas show EBV DNA.

### 3.4.1 Prognosis

The prognosis of NHL in AIDS patients is very poor, and survival of patients is counted in months. Treatment involves chemotherapy with various cytotoxic drugs.

## 3.5 Periodontal Diseases

Periodontal diseases associated with HIV infection and AIDS are still the subject of controversy. In the classification system drawn up by the European Community (EC 1993), it was stated that, in addition to the specific forms of periodontal disease, it should be appreciated that chronic marginal gingivitis and adult periodontitis can occur in patients with HIV infection. The clinical appearances of these conditions may, however, be altered or exaggerated as a result of immunosuppression. Periodontal diseases are considered by some as the same periodontal diseases seen in normal patients with some exaggerated features and by others as specific diseases particular to HIV infection or AIDS.

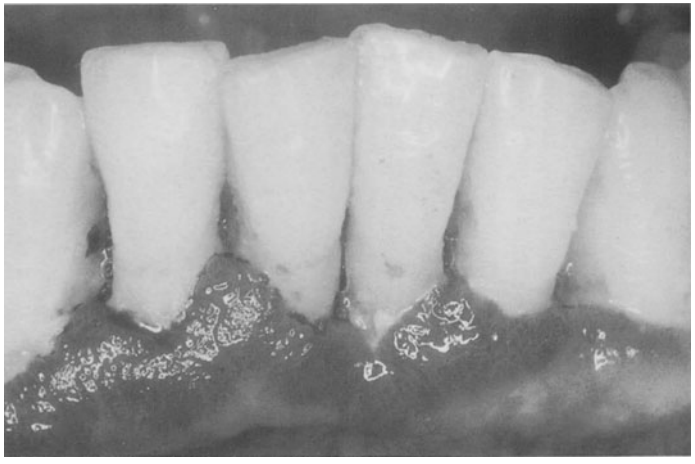
### 3.5.1 Linear Gingival Erythema

Linear gingival erythema (LGE) is described as a distinct, firey-red band along the margin of the gingiva (Fig. 5a). The amount of erythema is disproportionally intense for the amount of plaque seen. No ulceration is present, and there is no evidence of pocket or attachment loss. There are currently no definite criteria, and LGE is diagnosed clinically. It has been suggested that a feature of LGE is that it does not respond well to oral hygiene measures and to the removal of dental plaque and calculus. The microbiology of this lesion is controversial; however, *Candida albicans* seems to be involved in the aetiology (Grbric et al. 1995). The specificity of LGE and its association to HIV infection and AIDS has been questioned by several clinicians; similar, if not identical changes have been observed in debilitated patients.

Biopsy is not recommended for making the definite diagnosis.

### 3.5.2 Necrotising (Ulcerative) Gingivitis

Destruction of one or more interdental papillae is a presumptive criterion of necrotising gingivitis. In the acute stage of the process, ulceration, necrosis and sloughing is seen with ready haemorrhage and a characteristic odour. As in all the



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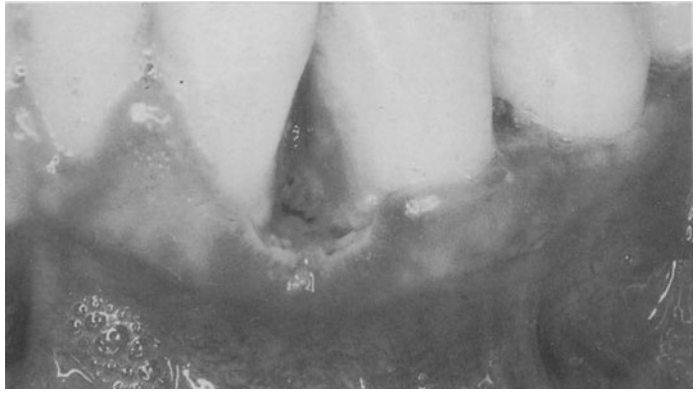

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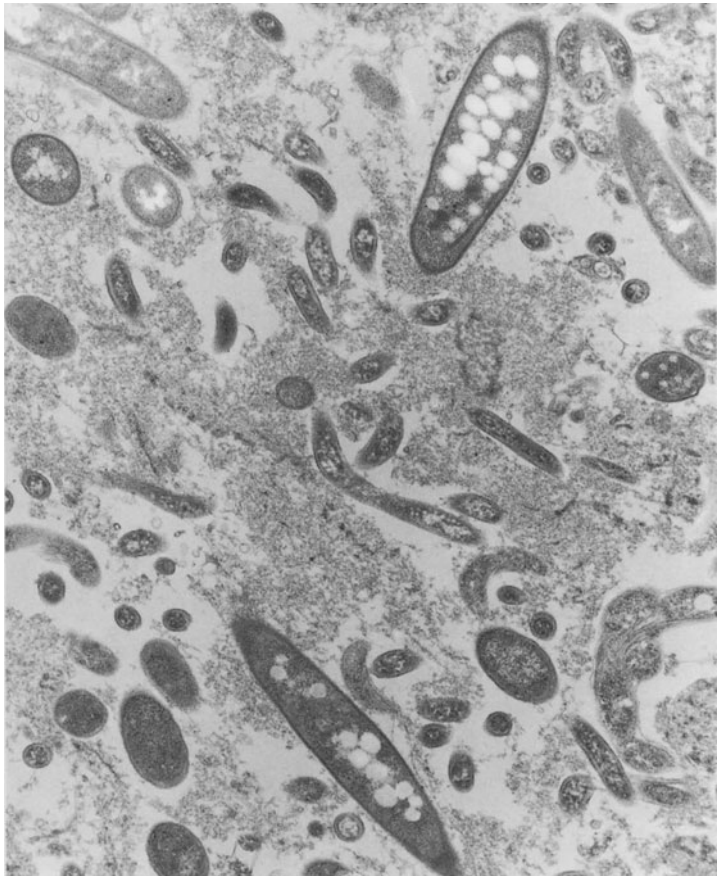

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**Fig. 5.** **a** Characteristic linear gingival erythema in a human immunodeficiency virus (HIV)-positive patient. **b** Necrotising gingivitis with characteristic loss of papilla in an HIV-positive patient. **c** Transmission electron micrograph of numerous spirilliform bacteria and cocci in a biopsy of necrotising gingivitis.

other periodontal diseases associated with HIV infection or AIDS, no definite criteria are available. Figure 5b shows a typical loss of papilla in an HIV-infected patients. Necrotising gingivitis has been reported in 5%–11% of HIV-infected individuals (Holmstrup and Westergaard 1994). Most patients only show necrotic tops of the interdental papilla.

### 3.5.3 Necrotising (Ulcerative) Periodontitis

Necrotising periodontitis is characterised by presumptive criteria such as soft tissue loss as a result of ulceration or necrosis. Exposure, destruction or segregation of bone is seen, and teeth may become loose. There may be severe pain. As in LGE and necrotising gingivitis, no definite criteria are known for necrotising periodontitis. A study carried out by the U.S. National Institute of Dental Research of 200 HIV-seropositive patients found that the type of periodontitis seen in these patients did not have unique or pathognomonic characteristics that could set it apart from the periodontal disease in HIV-seronegative patients (Riley et al. 1992). The microbiology of both necrotising gingivitis and necrotising periodontitis show micro-organisms such as *Borrelia*, gram-positive cocci, $\beta$-haemolytic streptococci and *Candida albicans* (Zambon et al. 1990). Figure 5c shows a thin section of a biopsy of necrotising periodontitis with numerous organisms invading the marginal gingival tissue.

### 3.5.4 Prognosis

Periodontal diseases in HIV infection and AIDS have been overestimated as to their prevalence and significance. As with periodontal diseases in non-immunocompromised patients, these lesions and diseases may be prophylactically avoided by appropriate oral hygiene measures, although rapid destruction of periodontal tissue has been observed. It must be stressed that the periodontal lesions are interrelated and that they are not individual characteristic entities. A necrotising stomatitis may well occur as a result of necrotising gingivitis or periodontitis. More severe lesions, however, seem to be rare.

## 4 Lesions Less Commonly Associated with Human Immunodeficiency Virus Infection (Group II)

Group II comprises some bacterial infections of mycobacterial origin and viral infections including herpes simplex virus, human papillomavirus and varicella zoster virus. In addition, other diseases such as NOS ulceration, salivary gland disease and melanotic hyperpigmentation are part of this group. Of particular interest are NOS ulcerations and human papillomavirus-induced oral lesions.

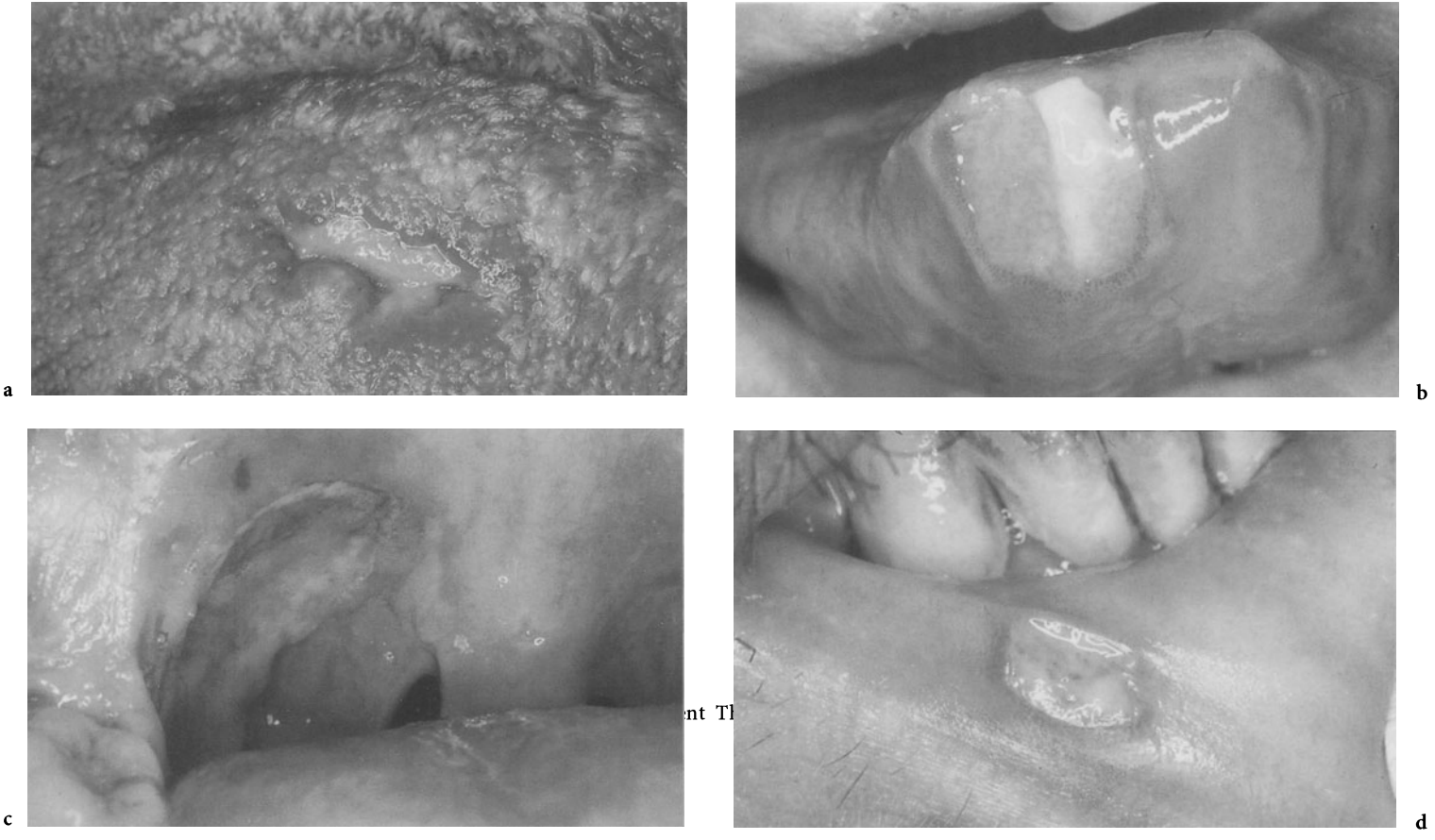


**Fig. 6.** **a** Small ulceration on dorsum of tongue which was shown to be an ulceration due to cytomegalovirus (CMV). Immunohistochemical staining for CMV is usually necessary to establish a correct diagnosis. **b** Large ulceration at the tip of the tongue associated with herpes simplex virus (HSV). This is an unusual location for herpesvirus-associated ulceration. **c** Ulceration not otherwise specified on the fauces and palate of an AIDS patient. Histology is nonconclusived Chronic recurrent ulcer on lipmucosa of an AIDS patient The characteristic clinical morphology of this type of aphthae is seen.

## 4.1 Ulcerations Not Otherwise Specified

Oral ulcerations are very common in the course of HIV infection and AIDS. They may be caused by fungal, bacterial and viral infections as well as by neoplasia (Fig. 6a–d). While an underlying cause may be found in most of the above-mentioned types of ulceration, this is not so in NOS ulcerations. These were called atypical ulceration in former classifications. NOS ulcerations are characterised by presumptive criteria, including those ulcerations with a predilection for the pharynx and palate which do not correspond to any of the recognised patterns of recurrent aphthous stomatitis (RAS). The definite criteria include histological features of a non-specific ulceration. Viral or bacterial cultures fail to identify a specific aetiological agent (EC 1993). NOS ulcerations have been described under a variety of names such as major aphthous-like ulcers or recurrent aphthous ulcers of the major type. In one study, 66% of 75 HIV-seropositive patients presented with the uncommon herpetiform and major types of ulcerations. Patients with this type of ulceration were significantly more immunosuppressed than those with minor or herpetiform recurrent aphthous ulcerations in that they had fewer $CD4^+$ and $CD8^+$ lymphocytes (MACPHAIL et al. 1991). NOS ulcerations are often painful and not responsive to treatment. They may persist for weeks. Treatment usually involves topical tetracyclines and steroids; however, resistant ulceration has also been treated with thalidomide.

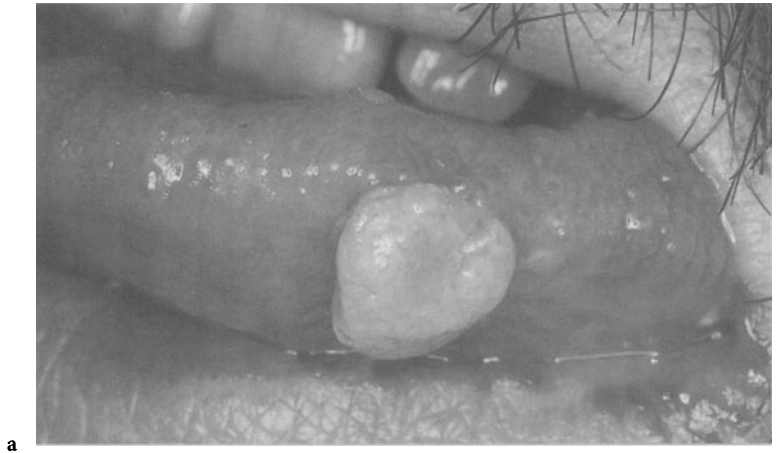
The aetiology and pathogenesis of NOS ulcerations is still not clear. Several mechanisms may be operative in the aetio-pathogenesis of such ulcers (REICHART 1992):

1. A directly or indirectly antibody-mediated mechanism
2. A T cell-mediated mechanism
3. Antibody-dependent cellular cytotoxicity
4. A natural killer (NK) cell-mediated mechanism
5. A specific or non-specific immunocomplex-mediated mechanism

The diagnosis is mainly based on clinical appearance and history of the ulceration. No reliable histopathological criteria have yet been found to characterise NOS ulceration. To a certain extent diagnosis is also made by exclusion of other causes, such as fungal, bacterial and viral agents. The prognosis is fair because the disease process may be considerably shortened with local or systemic steroids and/or thalidomide. Recurrences, however, may occur.

## 4.2 Human Papillomavirus Infections

Epithelial verruca-like proliferations of the oral mucosa have been reported in HIV-positive individuals. Verruca vulgaris, condyloma acuminatum and focal epithelial hyperplasia (FEH) have been described. Lesions may either occur as tiny proliferations or may involve large areas of the oral mucosa (Fig. 7a). Lesions are associated with human papillomavirus, especially type 7 or other types including



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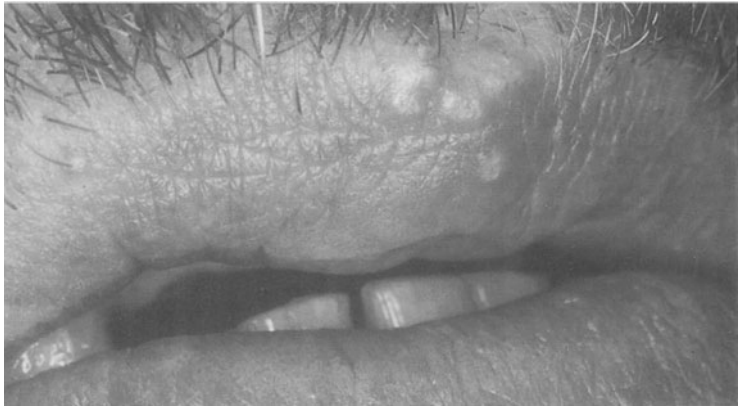

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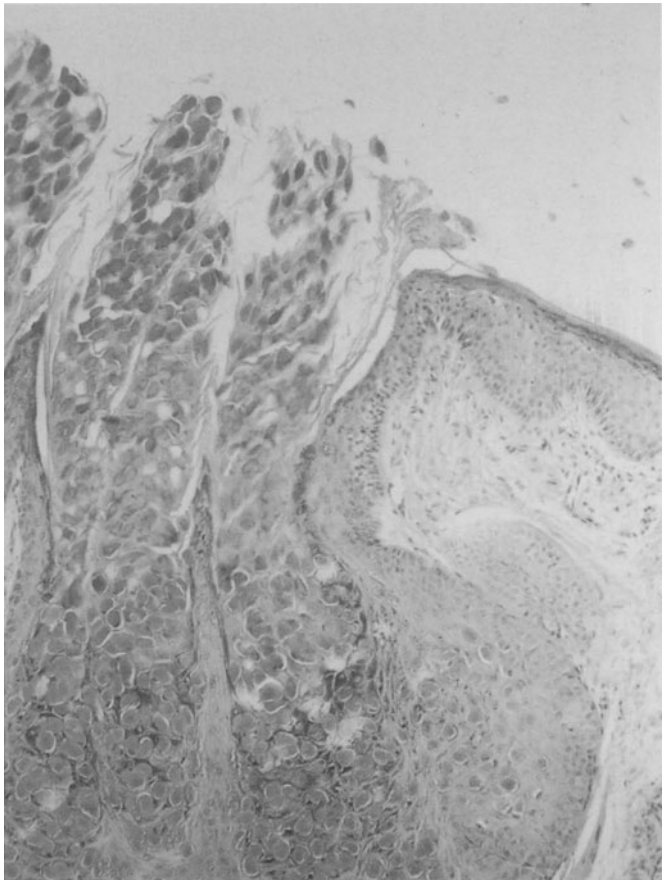

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**Fig. 7.** **a** Human papillomavirus (HPV)-associated proliferation at the lateral border of tongue of a human immunodeficiency virus (HIV)-positive patient. In this case the lesion had a more papilloma-like appearance. **b** On the upper lip of this HIV-positive patient, the characteristic lesions caused by molluscum contagiosum are seen. **c** Histological section of molluscum contagiosum-associated lesion, showing characteristic eosinophilic intracytoplasmic inclusion bodies These cells show hyperplastic growth and form large continuous lobules H&E ×200

13, 18 and 42. The clinical appearance of human papillomavirus-associated oral mucosa lesions is the same in immunocompromised as in immunocompetent subjects (for a review, see Syrjänen 1995). The histopathological criteria are also identical. Human papillomavirus is not associated (as was initially suspected) with HL. Cryosurgery, laser therapy or surgical excision are usually used in treatment of oral papillomatous lesions.

### 4.3 Necrotising Stomatitis

Necrotising stomatitis is characterised by presumptive criteria such as localised, acute, painful ulcerative necrotic lesions of the oral mucosa that expose underlying bone or penetrate or extend into neighbouring tissues. These lesions may extend from areas of necrotising periodontitis. Definite criteria show histological features of non-specific ulceration. Microbiological studies fail to identify a specific aetiologic agent. Extensive noma-like cases of necrotising stomatitis have been described, and perforation of facial skin may be observed in such cases. As has already been discussed, necrotising stomatitis is not an entity in itself, but is usually determined by gingival or periodontal necrosis. The prognosis in such extensive cases of necrotising stomatitis is poor, and the disease may be life-threatening.

### 4.4 Human Immunodeficiency Virus Associated Salivary Gland Disease

HIV-associated salivary gland disease (HIV-SGD) encompasses non-neoplastic changes of the salivary glands with enlargement of one or more of the major salivary glands with or without xerostomia. Diseases such as benign lymphoepithelial lesion, cystic lymphoid hyperplasia of the parotid gland, lymphadenopathy of the parotid gland, parotid swelling or enlargement, sicca complex syndrome and Sjögren's syndrome are considered to belong to HIV-SGD (Schiödt 1992). HIV-SGD appears to be rather uncommon in adult HIV patients (0%–0.8%), but it has been observed in 0%–58% of HIV-infected children (Schiödt 1992). The age distribution of patients with HIV-SGD is characterized by two groups: children born of HIV-infected mothers and adults between 20 and 60 years of age. Men are affected in over 90% of cases. Intravenous drug users are affected in 61% and homosexual men in 39% of cases. The main symptom is swelling of one or more of the major salivary glands, most often the parotid. A total of 91% of patients with HIV-SGD or Sjögren's syndrome-like conditions complained of a dry mouth. The parotid gland is affected in more than 90% of patients, while the submandibular gland is only affected in 2%. Diagnostic procedures involve measurement of salivary flow rates, labial salivary gland biopsy, eye examinations and serological examination for antinuclear antibodies, rheumatoid factor and Sjögren's syndrome antibodies A and B. HIV-SGD has a characteristic appearance on computed tomography (CT) and magnetic resonance imaging (MRT).

Multicentric cysts within salivary glands are characteristic. Histopathology uniformly reveals lympho-epithelial lesions or cysts. The lesion is composed of hyperplastic intraparotid lymph nodes or a lymphocytic infiltrate within the salivary gland tissue or both. Epimyoepithelial islands are seen within the lymphoid tissue, and a cystic lumen is seen centrally. The inflammatory infiltrate is dominated by $CD8^+$ cells. Labial salivary gland biopsy shows focal sialadenitis comparable to Sjögren's syndrome, with a predominance of $CD8^+$ cells. The prognosis of HIV-SGD is favourable, although no definitive treatment has so far been described. The pathogenesis of HIV-SGD is unknown.

## 5 Lesions Seen in Human Immunodeficiency Virus Infection (Group III)

Group III of the classification (EC 1993) comprises rare diseases and lesions. Bacterial infections caused by *Enterobacteriaceae* and other agents have been described. Fungal infections comprise those of rare diseases such as cryptococcosis, geotrichosis, histoplasmosis, mucormycosis and others. Viral infections include CMV and molluscum contagiosum (Fig. 7b,c). Lesions in group III include drug reactions, epithelioid angiomatosis and neurological disturbances of facial nerves.

All of these lesions are anecdotal and have only been described in rare instances. Their occurrence may not necessarily be associated with HIV infection. Due to geographical differences, other types of rare infections may be included in group III in the future. One example is penicilliosis, which is rare in Europe and North America, but rather prevalent as an oral lesion in South-East Asia, particularly in Thailand.

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# Extranodal Non-Hodgkin's Lymphomas of the Oral Cavity

R.C.K. JORDAN and P.M. SPEIGHT



## 1 Introduction

Lymphomas are malignant neoplasms of component cells of lymphoid tissues. A broad division of the group into Hodgkin's disease and non-Hodgkin's lymphoma (NHL) is widely accepted. Hodgkin's disease is primarily a nodal disease characterised by the presence of Reed-Sternberg cells and a lymphoid stroma composed of large numbers of non-neoplastic cells (BONADONNA et al. 1989). The condition rarely affects extranodal sites and will not be discussed here.

NHL comprise a heterogeneous group of lymphoid neoplasms with a spectrum of behaviour ranging from relatively indolent to highly aggressive and potentially fatal (SALHANY and PIETRA 1993). The classification of this diverse group of diseases has been based on the study of lymphomas arising in lymph nodes. Moreover, the biology and natural history has in large part been based upon the study of nodal disease, and only recently has more attention been directed at lymphomas arising at extranodal sites. Up to 40% of all NHL arise at extranodal sites, and the most common location is the gastro-intestinal tract. In the West, they most commonly occur in the stomach, but in the Middle East the intestine is the most common location. The head and neck is the second most common site for extranodal NHL, with the majority of cases arising in Waldeyer's ring, a band of lymphoid tissue which encircles the oropharynx.

Similar to lymphomas arising in lymph nodes, B cell lymphomas are the most common phenotype at extranodal sites (REGEZI et al. 1991). A spectrum of B cell lymphomas occurs in the head and neck with a wide range of biological behaviour.

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Although most are diffuse, large cell lymphomas, other types are seen in specific sites and populations of patients. These include Burkitt's lymphoma (BL), which occur in the facial bones of young patients, and T cell lymphomas, which occur in the nasofacial region, producing the clinical condition termed "midline lethal granuloma" (RATECH et al. 1989). Within the salivary glands, most lymphomas arise within lymph nodes embedded in the salivary tissues (GLEESON et al. 1986) and have clinical and histological features similar to lymphomas arising in lymph nodes at other sites. Lymphomas may also arise within the salivary gland parenchyma and resemble those arising in mucosa-associated lymphoid tissues (MALT) (ISAACSON 1992). This group of tumours is genotypically and phenotypically unique and is characterised by a relatively long natural history. Others such as plasmacytoma and BL show a striking predilection for primary osseous involvement.

The clinical presentation of lymphomas of the oral regions varies with their site of origin and tumour type, but most patients present with a swelling or ulcerated mass which can bear striking resemblance to the most common oral malignancy, squamous cell carcinoma (HOWELL et al. 1987). Pain or dysphagia is the next most common symptom, but systemic manifestations are rare (MCGURK et al. 1985) The characterisation of specific lymphoma types is important, because staging procedures and therapy may differ for each type. The only reliable method to distinguish and characterise these lesions is by biopsy coupled with immunological studies of biopsy tissue (ROONEY and RAMSAY 1994).

This chapter will review a number of extranodal NHL which occur in the oral cavity, including specific subtypes that affect Waldeyer's ring, salivary gland and the nasofacial region.

## 2 Classification

The classification of lymphoid neoplasms continues to be a source of controversy. At least eight classifications have been proposed over the past 30 years, but none have gained universal acceptance (STEIN 1995). Currently, only two classifications are widely used, the Kiel system in Europe and the Working Formulation for Clinical Usage in North America (ROSENBERG 1994).

The basis for many systems has been the morphological and immunological resemblance of lymphoma cells to their non-neoplastic lymphocyte counterparts. LUKES and COLLINS (1977) proposed a classification scheme based on B or T cell lineage of the neoplastic lymphocytes. A similar system based on functional lineage was proposed by Lennert (LENNERT et al. 1975; LENNERT and FELLER 1990; Table 1) and further divides lymphomas into groups based on clinical behaviour.

A number of other classifications have flourished based on varying contributions of cell morphology, immunology and clinical behaviour. The Working Formulation is based on the placement of lymphomas into prognostic groups based

**Table 1.** Updated Kiel classification for lymphomas (STANSFELD et al. 1988; LENNERT and FELLER 1990)

| B cell lymphomas | T cell lymphomas |
|---|---|
| **Low grade** | **Low grade** |
| Lymphocytic | Lymphocytic |
| Chronic lymphocytic leukaemia | Chronic lymphocytic leukaemia |
| Prolymphocytic leukaemia | Prolymphocytic leukaemia |
| Hairy cell leukaemia | Small, cerebriform |
| Lymphoplasmacytic/cytoid (LP immunocytoma | Mycosis fungoides, Sézary syndrome |
| Plasmacytic | Lympho-epithelioid (Lennert's lymphoma immunocytoma) |
| Centroblastic/centrocytic follicular ± diffuse, diffuse | Angio-immunoblastic (angioimmunoblastic lymphadenopathy, lymphogranulomatosis X) |
| Centrocytic | T zone lymphoma |
| | Pleomorphic, small cell (HTLV-1$^+$ or HTLV-1$^-$) |
| **High grade** | **High grade** |
| Centroblastic | Pleomorphic, medium-sized and large cell (HTLV-1$^+$ or HTLV-1$^-$) |
| Immunoblastic | Immunoblastic (HTLV-1$^+$ or HTLV-1$^-$) |
| Large cell anaplastic (Ki-1$^+$) | Large cell anaplastic (Ki-1$^+$) |
| Burkitt's lymphoma | Lymphoblastic |
| Lymphoblastic | |
| **Rare types** | **Rare types** |

HTLV, human T lymphotrophic virus.

on morphological patterns of the tumour and the cytology of neoplastic cells. Although originally designed as a mechanism to translate between differing lymphoma classifications, the Working Formulation is now used as the primary classification system in many North American institutions. Since its introduction, it has been criticised for its poor immunological basis, its grouping together of heterogenous diseases and its omission of a number of important subtypes of lymphoma (ROSENBERG et al. 1982).

Most classifications of lymphomas have been based mainly on lesions arising within lymph nodes. Their application to extranodal lymphomas has been more problematic, since a number of entities which arise in these sites do not have obvious nodal counterparts and have not found their place in current schemes. The newest system for lymphoma classification, the Revised European American Lymphoma (REAL) scheme, proposed by the International Lymphoma Study Group, has attempted to remedy this situation (CHAN et al. 1994; HARRIS et al. 1994). This scheme divides lymphomas into T and B cell groups and includes a number of entities which arise at extranodal sites. However, it has received some criticism for its lack of clinical correlation and strong reliance on immunophenotyping to classify the conditions (ROSENBERG 1994; LENNERT 1995). Although it includes a number of newer lymphoma types, it remains to be seen whether this classification will gain universal acceptance.

## 3 Staging

The importance of proper staging for patients with lymphoma in the oral region cannot be overemphasised. Staging serves a number of important purposes, including the determination of the type and extent of therapy, the overall prognosis for the patient and the potential complications associated with the disease (BONADONNA et al. 1989).

The Ann Arbor method, although initially designed to stage Hodgkin's disease, is now widely used for NHL. Patients are generally assigned a stage between I and IV, depending on the site and extent of their tumour (Table 2). In addition, they are classified as having A- or B-type symptoms, depending on the presence of constitutional symptoms associated with the tumour (BONADONNA et al. 1989).

The staging procedure often differs for the type and site of lymphoma within the head and neck region. Gastro-intestinal assessment is performed for lymphomas of Waldeyer's ring, since these tumours are often accompanied by gastro-intestinal involvement. Lymphomas of MALT tend to remain localised for prolonged periods and have a relatively indolent clinical course hence less extensive investigation is often required. Assessment of the central nervous system (CNS) is performed for lymphomas of the nose and paranasal sinuses and for lymphoblastic lymphoma and undifferentiated types. Bone marrow biopsy is generally performed for all extranodal lymphomas of the head and neck, but staging laparotomy is rarely undertaken, since the yield is low (COBLEIGH and KENNEDY 1986).

## 4 B Cell Lymphomas

### 4.1 Lymphomas of the Salivary Gland Including Mucosa-Associated Lymphoid Tissue

The incidence of lymphomas arising in salivary glands is controversial, since most lymphomas arise in lymph nodes embedded within the salivary tissues, whilst

**Table 2.** Ann Arbor staging system for non-Hodgkin's lymphoma

| Stage | Definition |
|---|---|
| I | Involvement of single lymph node region or of a single extranodal organ or site ($I_E$) |
| II | Involvement of two or more lymph node regions on the same side of the diaphragm, or localised involvement of an extranodal site or organ ($II_E$) and one or more lymph node regions on the same side of the diaphragm |
| III | Involvement of lymph node regions on both sides of the diaphragm, which may also be accompanied by localised involvement of an extranodal organ or site ($III_E$) or spleen ($III_S$) or both ($III_{SE}$) |
| IV | Diffuse or disseminated involvement of one or more distant extranodal organ with or without associated lymph node involvement |

others arise within the salivary gland parenchyma (GLEESON et al. 1986). The former group are similar to lymphomas arising within lymph nodes at other sites and will not be discussed here. It is increasingly being shown that lymphomas which arise in salivary gland parenchyma resemble those which develop in MALT. This term defines a group of unencapsulated lymphoid tissues adapted to protect mucosae exposed to the external environment (ISAACSON and WRIGHT 1983). The best characterised MALT is in the gastro-intestinal tract represented by Peyer's patches. In contrast to lymph nodes, where antigens are exposed to lymphoid tissue via the afferent lymphatics, in MALT antigens access B cells across an epithelial surface. Antigen stimulation of B cells results in the formation of immunoglobulin A (IgA) blast cells, which leave Peyer's patches through efferent lymphatics. These cells freely circulate and then return to the MALT as memory B cells or plasma cells through a poorly understood homing mechanism (PALS et al. 1989).

ISAACSON and WRIGHT (1983) were the first to describe a low-grade B cell lymphoma of the gastro-intestinal tract which recapitulated the features of MALT. These features were later described in a number of lymphomas arising in other sites, including the thyroid (ISAACSON and WRIGHT 1984), thymus (TAKAGI et al. 1992), salivary gland (HYJEK et al. 1988), conjunctiva (WOTHERSPOON et al. 1993), Waldeyer's ring (PAULSEN and LENNERT 1994), kidney (PARVEEN et al. 1993) and lung (LI et al. 1990).

One of the difficulties with the MALT lymphoma concept is that most do not arise at sites where MALT is most abundant, specifically Peyer's patches. Lymphomas of MALT tend to arise most commonly in the stomach, a site usually lacking lymphoid tissues. Furthermore, many cases of MALT lymphomas in the stomach arise in the setting of *Helicobacter pylori*-associated chronic gastritis. This bacteria can be identified in almost all cases of gastric lymphomas of MALT. It was thus proposed that these lymphomas arise in acquired MALT, including *H. pylori* gastritis and autoimmune diseases such as Hashimoto's thyroiditis and Sjögren's syndrome (SS). The prerequisite for the development of acquired MALT is reactive, chronic inflammation at a mucosal site (ISAACSON 1990). The development of MALT lymphoma in the major salivary glands is preceded by a salivary lympho-epithelial lesion (SLEL) (HYJEK et al. 1988; JORDAN and SPEIGHT 1996). This lesion usually arises in association with SS, although it may be seen in other auto-immune disorders and may occasionally may arise de novo. The risk of lymphoma development in SS is high, estimated to be 44 times that of the general population (KASSAN et al. 1978).

The clinical presentation of MALT lymphomas differs from other low-grade B cell lymphomas and resembles a chronic inflammatory process rather than a neoplasm (ISAACSON 1992). In contrast to nodal B cell lymphomas, they tend to remain localised for long periods and are late to disseminate. When spread does occur, it is usually to local lymph nodes, with dissemination to bone marrow being an uncommon and late event. Moreover, their clinical course is relatively indolent, and they generally respond to local measures such as surgical excision (ISAACSON 1992). This is in contrast to other low-grade B cell lymphomas, which are essentially incurable. Evolution of low-grade lymphomas of MALT to a high-grade lymphoma is well recognised, but although the prognosis is less favourable, it is

still better than for other high-grade B cell lymphomas (BATEMAN and WRIGHT 1993).

All low-grade lymphomas of MALT show similar histopathological features, irrespective of their site. The tumour is composed of a monotonous population of small to medium-sized lymphocytes which often have irregular nuclei and resemble centrocytes. Although these have been termed centrocyte-like cells (CCL cells), they can show a spectrum of morphology from resembling lymphocytes to monocytoid (ISAACSON and NORTON 1994; Fig. 1). In the salivary glands, lesions develop in salivary lympho-epithelial lesions (Fig. 2) and show "proliferation areas" of CCL cells, which typically invade and destroy the epithelium to form lympho-epithelial lesions which can be few or extensive (Fig. 3; BATEMAN and WRIGHT 1993). Monotypia can often be identified in these proliferation areas, which are considered to represent malignant lymphoma even in the absence of lymph node involvement (SCHMID et al. 1982). A large proportion of tumour cells may show plasmacytoid morphology, which in some cases can be so extensive as to resemble a plasmacytoma (WRIGHT 1994; Fig. 1b). The tumour cells begin in the marginal zone of MALT and gradually expand around reactive lymphoid follicles. With time, the neoplastic CCL cells may infiltrate reactive follicles in one of three patterns termed follicular colonisation (ISAACSON et al. 1991). Occasionally, this can give the tumours a vague nodularity, which can lead to a misdiagnosis of a follicular lymphoma (WRIGHT 1994).

The B cells of MALT lymphomas phenotypically express surface and cytoplasmic immunoglobulins, usually IgM, and show light-chain restriction. They usually express CD35 and CD21, but are CD10, CD23 and CD5 negative (ISAACSON 1993). The lack of CD5 is useful in differentiating the condition from mantle cell lymphomas, which invariably express this marker (ISAACSON 1992). Like follicle centre cell lymphomas, low-grade MALT lymphomas express the bcl-2 protein, but they consistently lack the chromosome translocation t(14;18) (WOTHERSPOON et al. 1990; DISS et al. 1995; JORDAN et al. 1995).

The term extranodal marginal zone B cell lymphoma has recently been proposed by the International Lymphoma Study Group as a pathologically more accurate designation for this group of lymphoid neoplasms (CHAN et al. 1994). Although it would appear that this better reflects the cell of origin of these tumours, for the time being the term MALT lymphoma is still more widely recognised and will continue to be used here.

A recently described condition termed monocytoid B cell lymphoma (MCBL) shares many similarities with lymphomas of MALT (ORTIZ-HIDALGO and WRIGHT 1992). MCBL occurs predominantly in lymph nodes, but a proportion has also been reported at extranodal sites, sometimes in association with SS (NGAN et al. 1991). When they occur at extranodal sites, they are indistinguishable from lymphomas of MALT (ORTIZ-HIDALGO and WRIGHT 1992). It is now thought that MCBL is a nodal, marginal zone lymphoma (CHAN et al. 1994) and represents the nodal equivalent of lymphomas of MALT. In many cases, a subclinical MALT lymphoma can be demonstrated at an adjacent mucosal or glandular site, such as the stomach or a salivary gland, suggesting that in some cases nodal MCBL may represent a secondary MALT lymphoma (NIZZE et al. 1991).

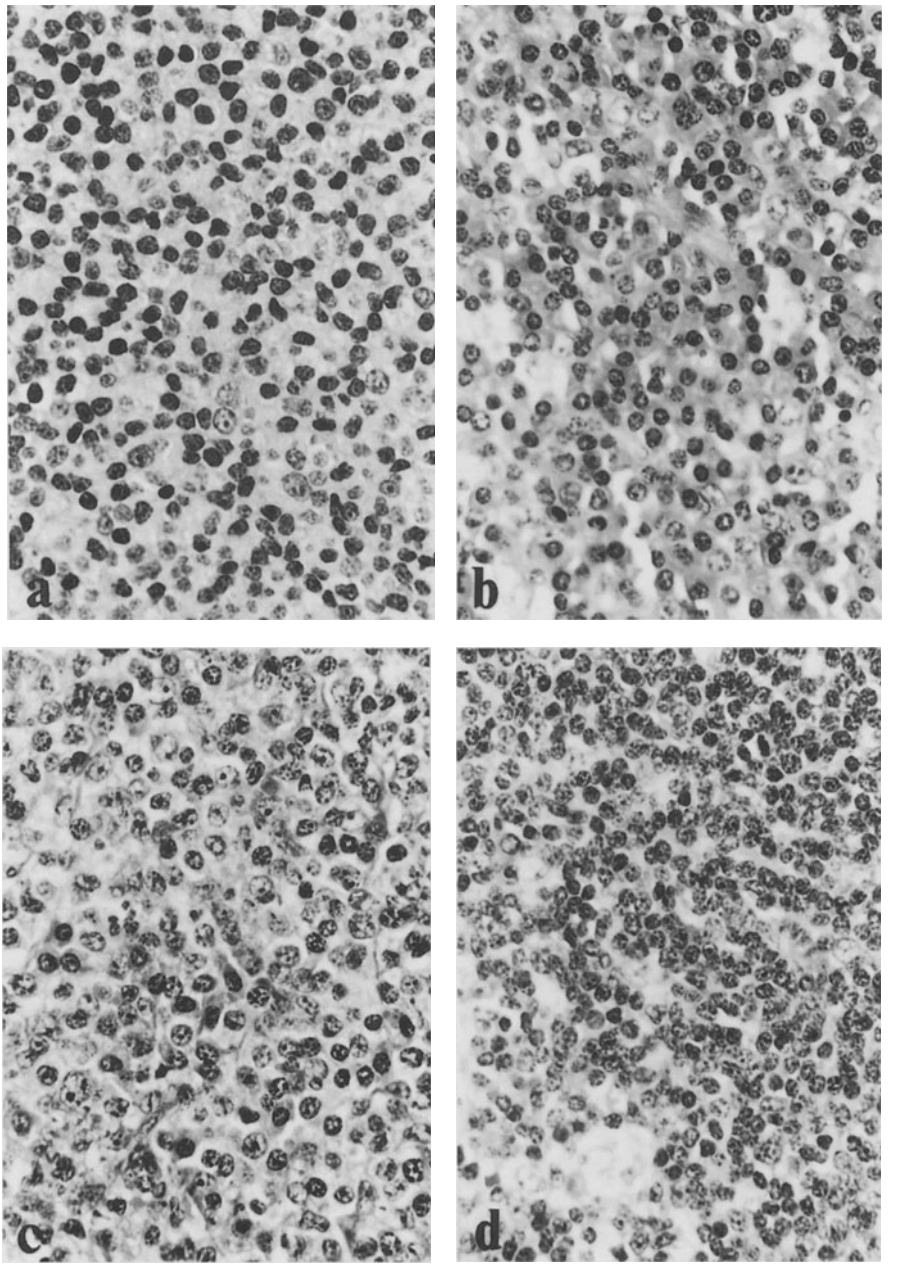


**Fig. 1a–d.** Cellular details from different mucosa-associated lymphoid tissue (MALT) lymphomas affecting the salivary glands, showing the variable morphology of the centrocyte-like cells. **a** The cells are quite small with irregular nuclei and resemble centrocytes. **b** Many cells show plasmacytoid differentiation. **c** Cells have abundant clear cytoplasm and resemble monocytoid B cells. **d** The cells resemble small lymphocytes. H&E, ×240

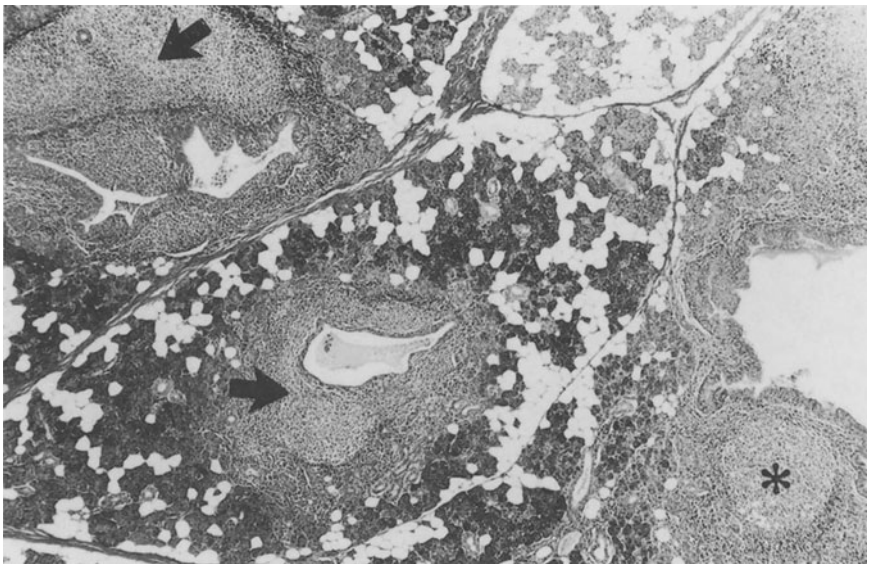

**Fig. 2.** An early mucosa-associated lymphoid tissue (MALT) lymphoma in a salivary lympho-epithelial lesion. Infiltrates of lymphocytes surround dilated ducts and islands of epithelium. In places follicle centres are seen (*asterisk*) adjacent to a duct, producing the typical MALT appearance. Elsewhere there are proliferation areas of clear centrocyte-like (CCL) cells. Those indicated (*arrows*) showed kappa light chain restriction on immunocytochemistry. H&E, ×15

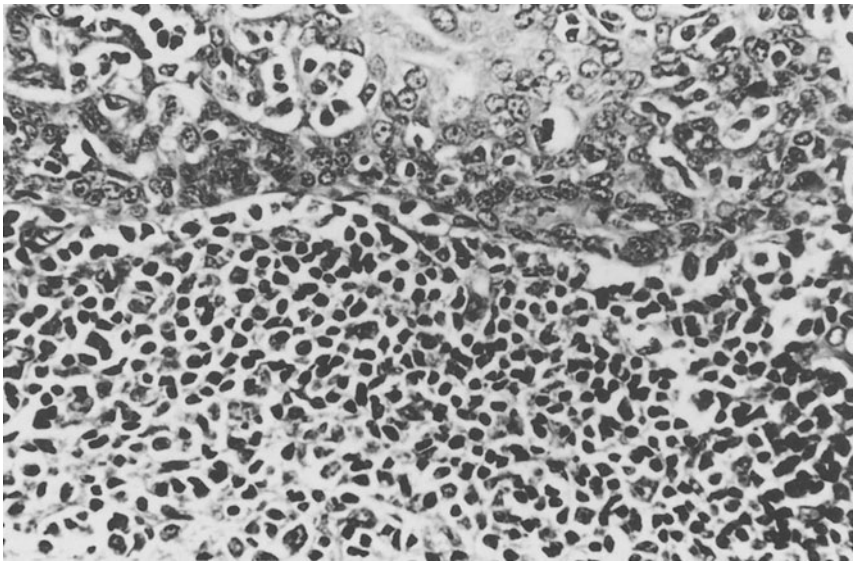

**Fig. 3.** A low-grade mucosa-associated lymphoid tissue (MALT) lymphoma from the parotid gland. Sheets of centrocyte-like cells have infiltrated and disrupted an island of proliferating epithelium to form a typical lympho-epithelial lesion. H&E, ×210

## 4.2 Lymphomas of Waldeyer's Ring

Waldeyer's ring is a band of lymphoid tissue which encircles the entrance of the aerodigestive tract and includes lymphoid tissues of the tonsils, nasopharynx and base of tongue. These tissues may represent a functional component of MALT, since they act as an interface between the systemic lymphoid tissues and the gut (WRIGHT 1994). Furthermore, studies have shown a morphological similarity between lymphoid tissues of Waldeyer's ring and those at other MALT sites, including a lack of sinusoids, a marginal zone of B lymphocytes and antigen presentation across an epithelial surface (MENARGUEZ et al. 1994). Although Waldeyer's ring shares many anatomical and functional similarities with MALT, the lymphomas arising at this site are more typical of lymphomas arising in lymph nodes (ISAACSON and NORTON 1994). The majority are centroblastic and centroblastic/centrocytic lymphomas.

However, Waldeyer's ring is still the second most common site for extranodal lymphomas after the gastro-intestinal tract. Most arise within the palatine tonsil, followed by the nasopharynx and then the base of tongue and soft palate (ECONOMOPOULOS et al. 1992). Multiple sites within Waldeyer's ring may be involved in about 4% of cases.

A total of 85% of lymphomas in Waldeyer's ring are high-grade B cell lymphomas, followed in order of frequency by extramedullary plasmacytoma and T cell lymphomas, including lympho-epithelioid lymphoma (Lennert's lymphoma) (BURKE and BUTLER 1992; ISAACSON and NORTON 1994; MENARGUEZ et al. 1994).

Histologically, high-grade lymphomas consist of cohesive sheets of pleomorphic lymphocytes and immunoblasts (Fig. 4) with a high rate of apoptosis and areas of necrosis. Only a minority of lymphomas at this site have been described as resembling lymphomas of MALT. PAULSEN and LENNERT (1994) reported that 12 of 329 lymphomas of Waldeyer's ring in their series were low-grade MALT lymphomas which predominantly affected the palatine tonsils. Histologically, two cases also contained high-grade elements. MENARGUEZ et al. (1994) reported only one MALT lymphoma in a series of 79 lymphomas of Waldeyer's ring. Three MCBL were also identified in this series, although they may now be classified as lymphoma of MALT.

The clinical and genotypic features of some high-grade lymphomas of Waldeyer's ring, including a good overall prognosis, epitheliotropism and lower bcl-2 protein expression, suggest that some of these tumours may represent high-grade MALT lymphomas. The diagnosis of a high-grade MALT lymphoma, however, requires the identification of low-grade areas within the same biopsy specimen. This can occasionally be difficult in small specimens, and it is not clear whether all series have been able to address this issue in all cases. It remains to be determined whether MALT lymphomas represent a larger proportion of lymphomas in Waldeyer's ring than has been previously reported.

Clinical staging is particularly important for lymphoma arising in Waldeyer's ring both from a therapeutic and prognostic standpoint. Lymphomas in this region are frequently associated with gastro-intestinal involvement, and a thorough

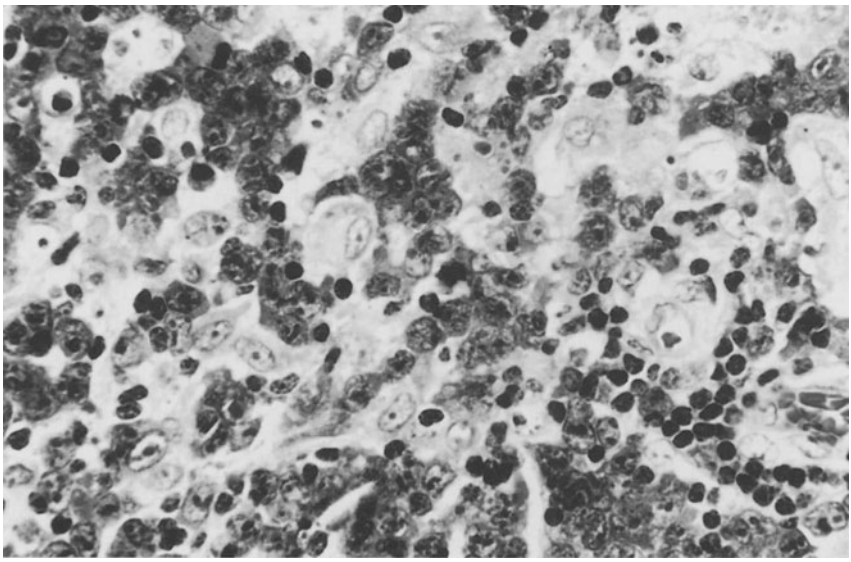

**Fig. 4.** High-grade B cell lymphoma. The tumour is composed of sheets of pleomorphic lymphocytes with many large immunoblasts. H&E, ×335

work-up will often result in upstaging of the patient's disease (MORTON et al. 1992).

## 4.3 Burkitt's Lymphoma

BL is a high-grade B cell lymphoma which primarily affects children and adolescents (BURKITT 1958). Two forms of the disease are recognised, an endemic type in tropical climates where malarial infection is also common and a sporadic form occurring in North America and Europe (PATTON et al. 1990). Involvement of the jaws is particularly common in endemic BL, with up to 50% of those affected having lesions of the maxilla or mandible. Multiple intra-oral sites can also be infiltrated simultaneously in up to one quarter of cases, and in up to one sixth of cases all four quadrants are affected simultaneously. Jaw lesions in sporadic BL are considerably less common than in the endemic form and occur in less than 6% of cases (BURKITT 1966). The small intestine and retroperitoneum are the most commonly affected sites in sporadic BL. A significant proportion of patients with BL will have bone marrow involvement at presentation, but leukaemic manifestations are rare (MINERBROOK et al. 1982).

The Epstein-Barr virus (EBV), a member of the herpes group of DNA viruses, has been identified in a large proportion of endemic BL. Only 10%–20% of cases of sporadic BL are associated with EBV infection (ANDERRSON et al. 1976). The EBV genome is not integrated in BL, but persists in a circular, episomal

state. Expression of EBV antigens is limited to EBV nuclear antigen (EBNA)-1, principally related to restricted promoter usage by the virus (SHAEFER et al. 1991).

Specific chromosome translations have been associated with BL. The translocation t(8;14) can be identified in up to three quarters of cases, juxtaposing the c-*myc* oncogene with the immunoglobulin heavy chain gene. Two other translocations have been described less frequently, but both involve translocation of the c-*myc* oncogene with either the kappa or the lambda immunoglobulin light chain gene. The translocation insertion sites differ in the endemic and sporadic forms of BL and are likely related to differences in B cell ontogeny in each form (PELICCI et al. 1986).

Microscopically, both forms of the disease show similar features, consisting of monomorphic sheets of densely packed, medium-sized neoplastic blast cells. The cytoplasm of the cells is deeply basophilic and often forms acute angles with neighbouring cells in well-fixed sections. The tumour has a very high mitotic rate, often with more than ten mitoses per high-power field, and it infiltrates widely through bone, occasionally involving the teeth (Fig. 5). Numerous macrophages containing cellular debris give the classical "starry sky" appearance to the tumour. Immunohistochemistry shows reactivity for many B cell markers, including CD19, CD20, CD22 and CD37. Over 90% of endemic BL express CD10, in contrast to variable expression in the sporadic type. Endemic BL does not express CD23, but expression can be identified in up to 50% of the sporadic form (ISAACSON and NORTON 1994).

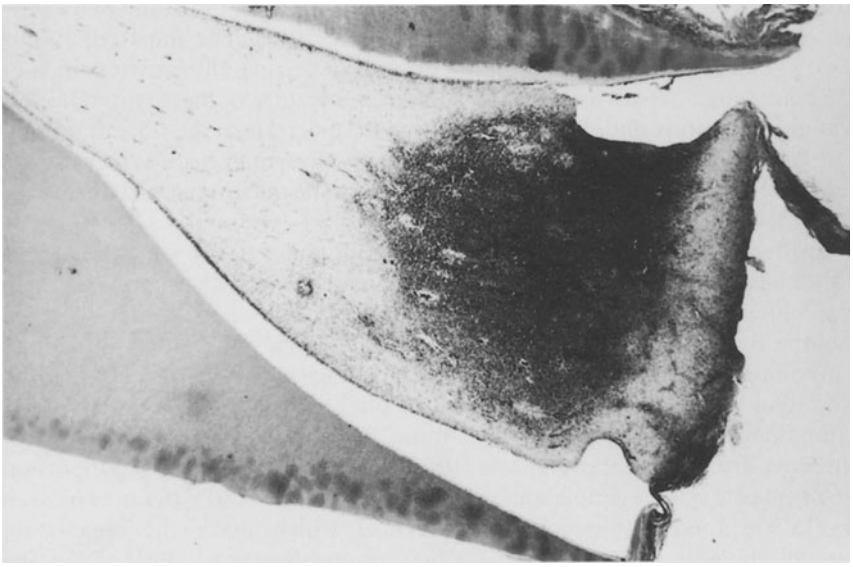

**Fig. 5.** This Burkitt's lymphoma in the jaw of a 6-year-old child has infiltrated into the pulp chamber of a partially formed incisor tooth. H&E, ×15

The tumour is particularly chemosensitive, with treatment often resulting in massive tumour lysis. Cure rates range from 54% to 59%, with relapses rare after 2 years (ZIEGLER 1981).

## 4.4 Lymphomas Associated with Human Immunodeficiency Virus Infection

The development of NHL has long been recognised as a rare complication of many congenital immunodeficiency states. The increase in organ transplantation coupled with immunosuppression techniques has also witnessed a marked increase in the development of many lymphoproliferative disorders. The development of lymphoma in the setting of the human immunodeficiency virus (HIV) is now seen as an important complication of acquired immunodeficiency.

NHL constitutes an AIDS-defining diagnosis in 3% of all patients (SERRAINO et al. 1992). It is a relatively late complication of HIV infection, with some lymphomas, particularly immunoblastic lymphoproliferations, occurring primarily when there is a marked depression of CD4-positive T cells (LEVINE 1992). All groups at risk for HIV infection are at risk of developing lymphoma, although some differences exist in the clinical and pathological features of individual risk groups (SERRAINO et al. 1992).

In contrast to lymphomas complicating other immunodeficiency states, up to 75% of those arising in HIV infection are extranodal and almost one fifth occur in the CNS (CARBONE et al. 1995). Sites of involvement are relatively distinct in AIDS-related lymphomas and include the CNS, ano-rectal region and the oral cavity (IOACHIM 1992). NHL account for 3% of all malignant tumours of the oral cavity in patients with HIV infection (SILVERMAN et al. 1986). The most commonly affected sites include the tonsillar region or gingiva as a rapidly growing mass or within bone causing tooth mobility. One important feature of these lymphomas is their rapid infiltration and the widespread extent of disease at the time of presentation (Fig. 6) and the common presence of systemic symptoms. Furthermore, a large proportion of patients will develop CNS and bone marrow spread during the course of their disease (IOACHIM 1992).

Most lymphomas complicating acquired immunodeficiency are of B cell origin, although T cell lymphomas also occur. Pathologically, there are two main types, immunoblastic lymphoma and Burkitt's-like (small non-cleaved) lymphoma (LEVINE 1992). Although both resemble their counterparts arising in non-immunosuppressed subjects, there are histological differences which make classification into conventional schemes difficult.

Immunoblastic lymphomas constitute the largest group of lymphoproliferative disorders that complicate HIV infection. Although a large proportion of the neoplastic cells resemble immunoblasts, many other cell types may be seen (Fig. 7), and there are genotypic differences which make the appellation immunoblastic lymphoma difficult. ISAACSCON and NORTON (1994) prefer the term polymorphous immunoblastic lymphoproliferation. This more accurately reflects the spectrum of pathology seen in these lesions, which includes a mixed

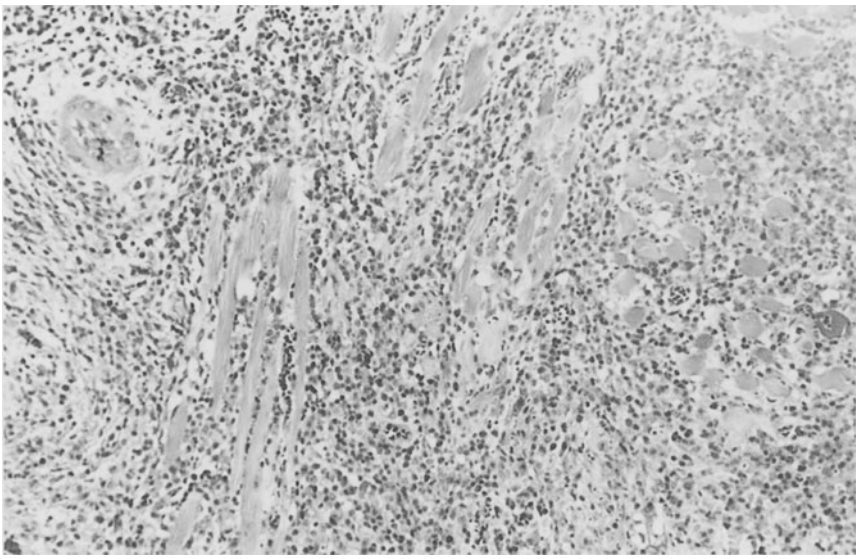

**Fig. 6.** High-grade B cell lymphoma associated with acquired immunodeficiency syndrome (AIDS). Pleomorphic immunoblasts and lymphocytes have infitrated deeply into the muscle of the tongue. H&E, ×85

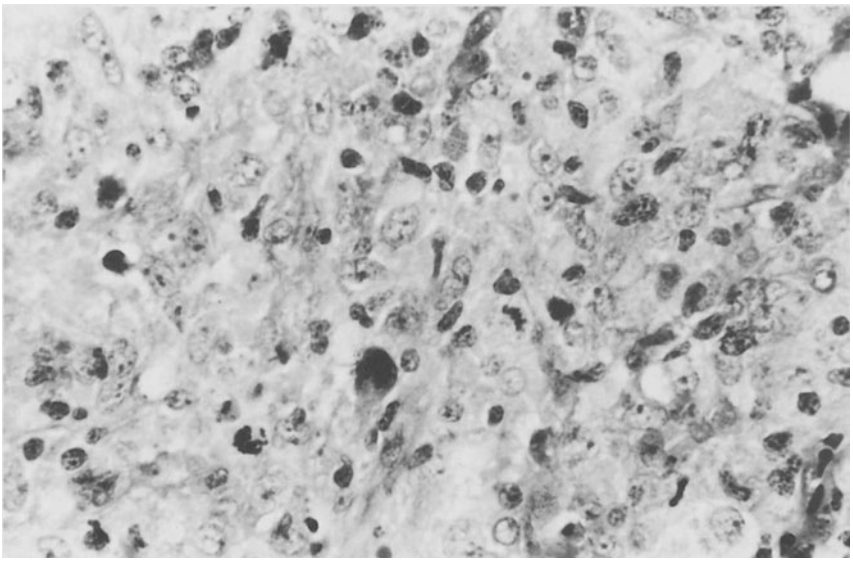

**Fig. 7.** A lymphoma of the gingiva associated with acquired immunodeficiency syndrome (AIDS). The tumour is composed primarily of pleomorphic immunoblasts with a high mitotic rate. Smaller lymphocytes, including inflammatory cells, are also seen. H&E, ×335

infiltrate of inflammatory cells with large pleomorphic immunoblasts, resembling florid infectious mononucleosis, and lesions with a predominance of centrocytes. Occasionally, more typical high-grade lymphomas, usually immunoblastic or anaplastic large cell lymphomas, may arise; these are rarely of T cell origin.

The other predominant lymphoproliferation to complicate HIV infection is a Burkitt's-like (small non-cleaved) lymphoma. Again, although these tumours bear a superficial resemblance to BL in non-immunosuppressed subjects, important histological differences also exist, including a greater degree of cellular pleomorphism and a high proportion of plasmacytic differentiation (ISAACSON and NORTON 1994).

The prognosis for lymphomas arising in HIV infection is generally very poor. The advanced stage at presentation, the aggressive behaviour of both low- and high-grade forms and the profound immunosuppression contribute to a poor outcome. The median survival for all patients with AIDS-associated lymphoma is 6.5 months (IRWIN and KAPLAN 1993).

## 4.5 Plasma Cell Tumours

The plasma cell dyscrasias include a number of diseases characterised by an expansion of a clone of immunoglobulin-secreting cells. These diseases are often characterised by the presence of immunoglobulin protein components in the serum or urine, termed M proteins. The spectrum of plasma cell dyscrasia includes multiple myeloma, solitary plasmacytoma of bone and extramedullary plasmacytoma. The biological behaviour of these conditions varies widely, although histologically all plasma cell tumours are similar and contain monotonous sheets of cells resembling plasma cells (Fig. 8). The cell population may vary from well-differentiated cells with an eccentric nucleus and basophilic cytoplasm to less-differentiated, atypical cells with occasional giant or multi-nucleated forms. Immunoblasts and plasmablasts are not seen, and there are very few inflammatory cells intermixed with the monotonous sheets of neoplastic plasma cells (WILTSHAW 1976). Immunohistochemical tests will show restriction of either kappa or lambda light chain and the presence of only one isotype of heavy chain, usually IgA or IgG (LENNERT and FELLER 1990; WAX et al. 1993). Occasional lesions contain extensive deposits of amyloid in the affected tissues.

The most common and important plasma cell dyscrasia is multiple myeloma. The condition is characterised by multiple osteolytic bone lesions, serum or urinary M proteins and a bone marrow biopsy showing greater than 10% plasma cell composition. Symptoms are related to infiltration of organs by neoplastic plasma cells and by the excessive production of immunoglobulins which have abnormal biochemical properties. Pathological fractures occur in 20% of patients. Advancing disease is associated with hypercalcaemia and renal failure. Bone marrow infiltration leads to anaemia, thrombocytopenia and leukocytopenia, with the latter resulting in an increased susceptibility to infection. Jaw lesions can be identified in 30% of cases of multiple myeloma and radiographically appear as non-corticated radiolucencies, more common in the mandible than maxilla. The posterior por-

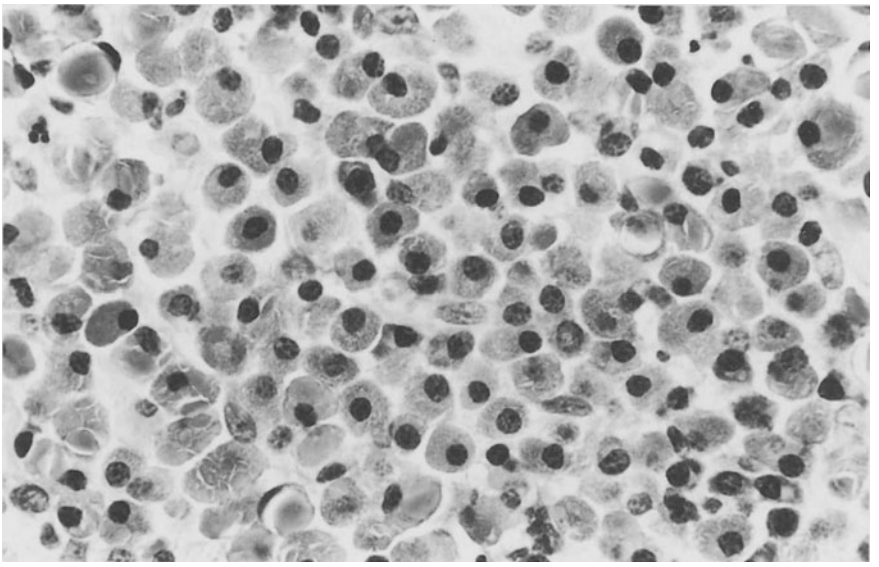

**Fig. 8.** A plasmacytoma from the gingiva. The tumour is composed of sheets of well-differentiated, although slightly atypical plasma cells. H&E, ×335

tions of the jaw are more commonly affected, as the marrow spaces are larger (Bruce and Royer 1953). The formation of amyloid from the aggregation of immunoglobulin light chain proteins is a common sequela of multiple myeloma and, when deposited in the tongue, can produce macroglossia (Reinish et al. 1994). Treatment of multiple myeloma is directed at reducing tumour burden and reversing complications of the disease, such as those related to renal failure. Single alkylating agent chemotherapy is the treatment of choice (Alexanian and Dimopoulos 1994).

A solitary focus of lytic bone destruction showing a plasma cell tumour without bone marrow involvement is termed solitary plasmacytoma of bone. Detection of M proteins in the serum or urine does not exclude the diagnosis of solitary plasmacytoma of bone. This lesion comprises 3% of all plasma cell neoplasms and is believed to represent a localised form of myeloma (Batsakis 1983). Involvement of the facial bones is rare and, when present, typically represents more disseminated disease. Progression to myeloma occurs in many patients, with almost one half having done so by 2 years, although long-term survival is also common (Corwin and Lindberg 1979). Solitary lesions are typically treated with radiation therapy supplemented by chemotherapy (Abemayor et al. 1988). When the disease is disseminated, it is treated in the same manner as multiple myeloma.

Isolated plasma cell tumours within soft tissues are termed extramedullary plasmacytoma. This does not include tumours that have arisen in bone and involved soft tissues secondarily, following perforation of the bone cortex. Over 80% of all extramedullary plasmacytomas arise in the upper respiratory tract and oral

cavity (WILTSHAW 1976). They form 4% of all non-epithelial neoplasms of the nose, nasopharynx and paranasal sinuses (FU and PERZIN 1979) and account for 20% of all extranodal lymphomas within the oral cavity (HANDLERS et al. 1986). The clinical appearance is of a dark-red, fleshy mass that is rarely ulcerated. Multiple lesions at other sites in the head and neck may be present in up to 20% of patients, and regional lymph nodes may be involved in up to 40%. Unlike multiple myeloma and solitary plasmacytoma of bone, wide dissemination is rare and typically shows no preference for active haematopoietic sites (WILTSHAW 1976). Many investigators have reported that the progression of extramedullary plasmacytoma to myeloma is distinctly uncommon, in contrast to the behaviour of solitary plasmacytoma of bone. One group has suggested, however, that progression to myeloma is equally frequent in both these tumours and that both represent a spectrum of the same disease (MEIS et al. 1987). Outcome and treatment options are not related to histological features of the tumour. Extramedullary plasmacytomas are radiosensitive, and regional control rates of 80% can be achieved (CORWIN and LINDBERG 1979).

## 5 Nasofacial T cell Lymphoma

Progressive, ulcerative destruction of the palate, nose and paranasal structures has long been recognised as a striking and potentially fatal condition. STEWART (1933) introduced the term mid-line lethal granuloma to describe the condition, but a number of other terms have been suggested, including polymorphous reticulosis, lymphomatoid granulomatosis, idiopathic destructive disease and mid-line malignant reticulosis (RAMSAY and ROONEY 1993). A number of diseases can produce destruction of mid-line facial structures, including Wegener's granulomatosis, infectious agents and lymphoma (HARRISON 1987), but it is now recognised that the majority of cases previously described as mid-line lethal granuloma represent a lymphoproliferative disorder, commonly a T cell lymphoma (RATECH et al. 1989). The term nasofacial lymphoma will be used here to describe the heterogeneous group of lymphoproliferative diseases that affect and destroy the mid-line palate, nose and paranasal structures (RAMSAY and ROONEY 1993).

Nasofacial lymphoma is typically a disease of adults. Nasal symptoms are often the most common presenting feature, with epistaxis occasionally present. Some patients may present early with swelling of the soft or hard palate. With time, there is evolution to frank ulceration, with destruction of the palatal and nasal tissues leading to the development of an oro-nasal fistula. Infection is often a late feature (RATECH et al. 1989).

The histological appearance of nasofacial lymphoma is characterised by the presence of varying amounts of granulation tissue and extensive coagulative necrosis. There is usually an inflammatory infiltrate consisting of a mixture of acute and chronic inflammatory cells. Intermingled with this are atypical lymphocytes which are strikingly pleomorphic and can range in number from only a few to extensive, monotonous sheets (Fig. 9). These cells are medium-sized or large with a clear cytoplasm and an irregular nuclear outline. Some have prominent nucleoli

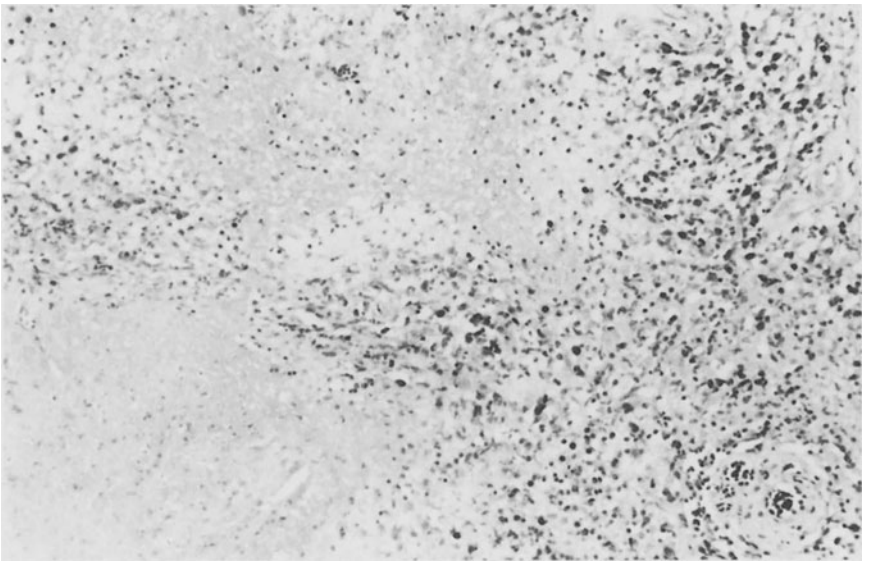

**Fig. 9.** A nasofacial T cell lymphoma. Sheets of pleomorphic lymphocytes are admixed with focal areas of necrosis. H&E, ×85

and may resemble immunoblasts. T cell lymphomas are the predominate type in the nasal region, and this is particularly true in Asian populations. Many of these cases resemble peripheral T cell lymphomas and phenotypically share many common features (Liepert et al. 1994). Angiocentricity (Fig. 10) and epitheliotropism are important and common histopathological features of nasofacial T cell lymphomas and provide evidence to support the contention that some of these tumours in the mid-face are related to angiocentric T cell lymphomas of the lung (Lipford et al. 1988).

Immunological staining shows that, in most cases of nasofacial lymphoma, the malignant cells express T cell markers, including CD45RO (UCHL-1), CD43 (MT1) and CD3 (Chan et al. 1987; Ramsay et al. 1988). A large number of macrophages can be demonstrated in the background, leading, in the past, to the erroneous conclusion that many nasofacial lymphomas were histiocytic in origin (Ramsay and Rooney 1993). A minority of nasofacial tumours are derived from B cells, and these will show the presence of B cell markers, with gene analysis showing heavy chain gene rearrangements (Maxymiw et al. 1992).

Without treatment, the relentless destruction of mid-face structures by the lymphomatous infiltrate can lead to death from haemorrhage or secondary infection. Typically, the condition is treated using chemotherapy, radiation therapy or a combination of both (Ratech et al. 1989). Reports of long-term survival vary, in part because of the confusion regarding the nature of the condition and the unclear terminology used in the past. Previous reluctance to clearly define the condition as a lymphoma has led to inadequate treatment

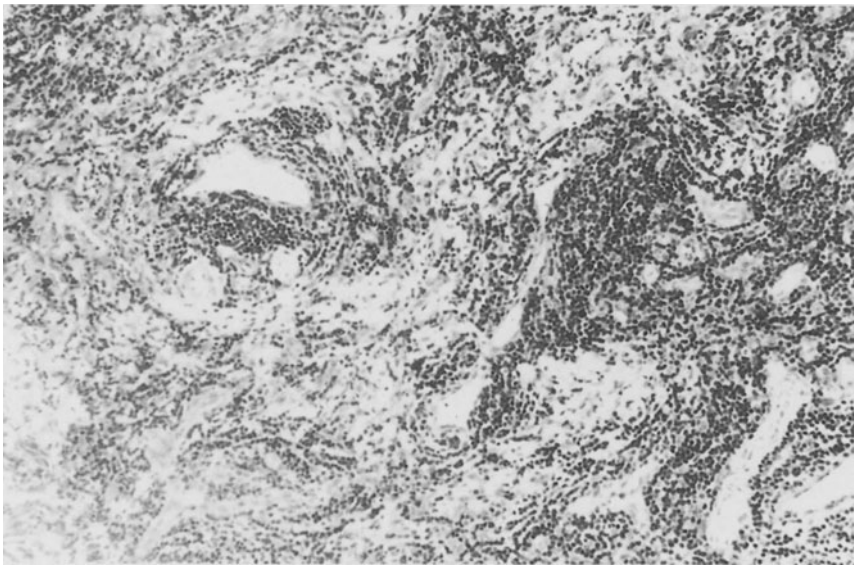

**Fig. 10.** A T cell lymphoma from the upper lip showing a prominent angiocentric pattern. H&E, ×85

and high recurrence rates. Overall survival from the time of diagnosis has been reported to be from 3 months to 14 years (Ramsay and Rooney 1993). More aggressive management in recent years has improved prognosis, with a 5-year disease-free survival of 78% for patients with early-stage lesions and 19% for those with more widely disseminated disease (Robbins et al. 1985). Others suggest that grade and stage of tumour do not affect survival for patients with nasofacial lymphoma, but this may reflect the heterogeneity of tumours that arise in this site (Ratech et al. 1989).

## 6 Conclusions

Extranodal lymphomas of the head and neck comprise a diverse collection of pathological entities. Although most are B cell neoplasms, other types are seen at specific sites, including MALT lymphomas arising in salivary glands and T cell lymphomas in the nasofacial area. The importance of identifying the subtle histological features of each, coupled with immunological and molecular biological studies of biopsy tissues, has been discussed. The clinical behaviour of these tumours varies greatly, as does management of the conditions. The requirement to properly categorise the specific lymphoma type further reinforces the need to have a thorough understanding of the biology of each condition.

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# Importance of Proliferation Markers in Oral Pathology

K.A.A.S.WARNAKULASURIYA and N.W. JOHNSON



## 1 Introduction

Disturbances in growth, including the carcinogenic process, are often linked with an increased rate of cell proliferation, usually combined with sustained hyperplasia (IVERSON 1992). As a result, over the past three decades, there has been considerable interest in markers of cell proliferation that can be used as indicators of the clinical aggressiveness of human neoplasms, additional to what can be judged from histology alone. The hope is that such markers will have value in prognosis and treatment planning. It is necessary at the outset to understand that the significance of "proliferative activity" depends on the number of cells undergoing programmed cell death or apoptosis (WYLLIE 1993) in a given compartment of cells. Aspects of cell proliferation have, however, been studied more extensively, and data taking apoptosis into account are limited.

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In this chapter we review, mainly in quantitative terms, the major parameters useful in understanding the kinetics of cell proliferation and look critically at the methodology used, with particular reference to epithelial systems and to oral mucosal disease. Data derived from many studies examining the cell kinetics of both oral and some other head and neck malignancies (particularly squamous cell carcinomas, SCC), potentially malignant lesions of the oral mucosa and a few other oral diseases of interest are presented. The usefulness of such investigations in understanding the pathogenesis of oral diseases and their potential value as diagnostic and prognostic markers in the discipline of oral pathology is addressed.

## 2 Methods of Studying Cell Proliferation

Methods of studying cell proliferation and growth in human neoplasms, together with their pitfalls, were reviewed by Quinn and Wright (1990), Hall and Levison (1990) and, with particular relevance to oral mucosa in health and disease, by Scragg and Johnson (1980a, 1982). Considerable advances in techniques have been reported since the latter report, and these are reviewed here.

Cell proliferation markers attempt to identify and measure the proportion of cells undergoing the particular phases of cell cycle. Studies involving dual parameter estimates over a period of time incorporate a measurement of time spent in each phase or in transit, thereby allowing not only assessment of the *state* of proliferation but also providing information about the *rate* of proliferation (Barnes and Gillett 1995).

The cell cycle is well described; the demonstrable stages are mitosis (M) and the DNA synthesis phase (S), during which replication of DNA in the nucleus occurs and is demonstrable by DNA labelling techniques. There is a time lapse ($G_2$ phase) between completion of DNA synthesis and beginning of mitosis. Similarly, daughter cells produced by mitosis do not start replicating DNA immediately, and this is referred to as $G_1$ phase. During this phase, cells may exit the cell cycle, and this "out of cycle" phase is referred to as $G_0$. Several cell cycle check points or restriction points exist mostly in $G_0/G_1$ phases, where a cell is able to integrate internal and external signals, allowing entry or exit of cells into and out of the cycle (Morgan 1992; Schneider et al. 1991). Factors which influence cell proliferation by operation through these check points are discussed in Sect. 5. Our knowledge on selection of markers to identify proliferating cells relates largely to those cells participating in the cell cycle; we do not yet have any means of accurately establishing any subpopulations of cells in resting $G_1$ or $G_0$ phases, so that the true growth fraction of a neoplasm cannot yet accurately be determined.

The majority of cell cycle estimates are known to be influenced by circadian rhythms (Pilgrim et al. 1963). Warnakulasuriya and MacDonald (1993, 1995a) have quantified the range of circadian variations expected in several cell kinetic parameters in human buccal mucosa. Human clinical studies need to control for this effect by appropriate sampling techniques.

## 2.1 Mitotic Counts

Mitoses can be counted in conventionally stained sections (Fig. 1), but the method identifies only a small fraction of the cycling population, because the time spent by cells in mitosis is relatively short – of the order of less than 1 h to a maximum of 2 h in oral epithelia – compared to other phases of the cell cycle. As mentioned earlier, the method allows only an assessment of the state of proliferation, and not the rate of proliferation. Standardizing mitotic counting techniques is important to achieve reproducibility. The selection of the counting field, criteria for identification of mitotic figures and other variables such as level of (microscope) focus all need to be taken into account (Aherne et al. 1977; Baak 1990). Delay in fixation, particularly of large specimens, can result in a reduction of mitotic figures, because cells move out of M phase ex vivo, thus lowering the count (Cross et al. 1990). Nevertheless, despite the methodological problems of assessing mitotic count in human neoplasms (Quinn and Wright 1990), when correctly measured and given as a fraction of interphase nuclear population, the *mitotic index* thus derived can be a useful marker of cell proliferation (Gillett et al. 1993).

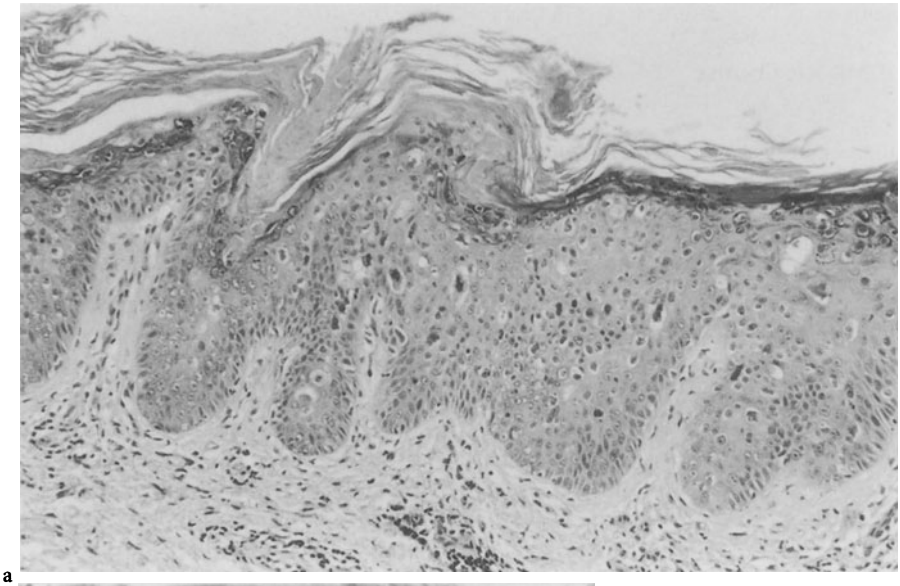
Mitotic arrest techniques provide more accurate data by increasing the number of figures available to be counted and introduce a longitudinal (time) component (Scragg and Johnson 1980b), but are rarely applicable to oral lesions in humans for ethical and safety reasons.

## 2.2 S-Phase Indices

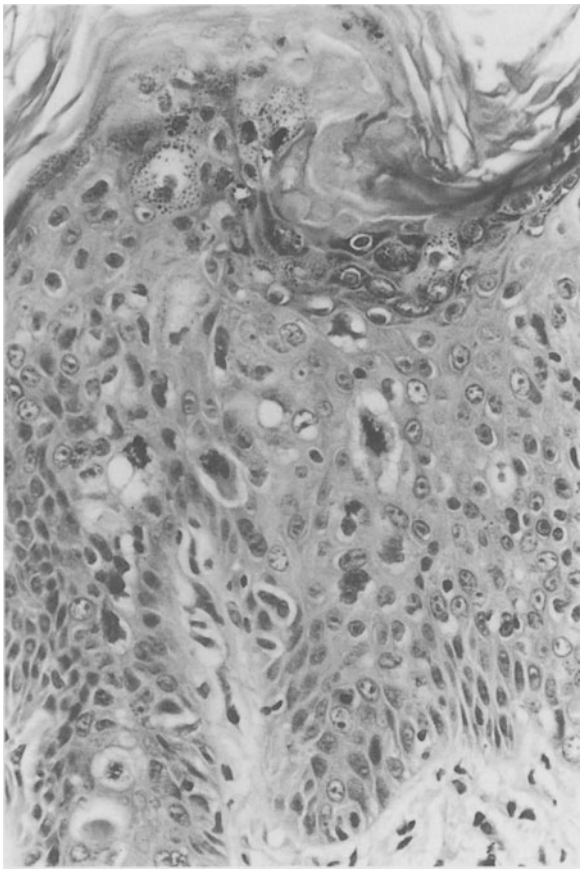
The most commonly used cell proliferation marker method in experimental studies has been to establish the proportion of cells in DNA synthesis (S) phase, referred to as the *labelling index* (LI), quantified on the basis of nucleic acid precursor incorporation using (usually) tritiated thymidine [$^3$H]dT or bromodeoxyuridine (BrdUrd).

### 2.2.1 Thymidine Labelling

Before the 1980s, cell proliferation status was mostly studied using [$^3$H]dT, and this technique continues to have a well-established role. Methods of in vitro incorporation into DNA by incubating freshly biopsied mucosal fragments over a short period of time (15 min) in a medium containing [$^3$H]dT have been standardised (Rennie et al. 1984) and can achieve reliable results. Autoradiographs prepared from pre-labelled tissues allow assessment of LI by counting labelled cells and expressing these as a proportion of 100 total cell population, or to a unit of surface length or of basement membrane length of the tissue, when covering epithelia are examined. In the past, linear measurements using microscope graticules or stereological techniques for morphometric estimates (Aherne and Dunhill 1982) have been used to denominate such indices; more recently, interactive computer



a



b

**Fig. 1a,b.** Increased mitotic activity in dysplastic oral epithelium from an in situ carcinoma. Numerous atypical mitotic figures and mitoses in superficial epithelial layers are apparent. **a** ×130. **b** ×330

systems with drawing devices and image digitisation have been a major advance (HAMILTON and ALLEN 1995).

A LI derived by a single-pulse label in vivo or in vitro allows an assessment of a static index, but with the introduction of double labelling techniques (WARNAKULASURIYA 1976) the time spent in S phase or the rate of cell influx and efflux can be calculated. This also allows an estimation of turnover time of a tissue or a compartment. In vitro incorporation of thymidine analogues has considerable methodological problems in that small pieces of fresh tissue trimmed to 1–2 $mm^3$ are required for incubation, and the representative nature of data derived from such small fragments of tissue has been questioned (LAMBERT 1986).

Data derived from pulse labelling also have the disadvantage – shared by many other similar techniques referred to later – that only cells in the actively dividing cell cycle are ascertained, and we do not know the actual compartment size of the growth fraction of the tissue which is represented by such data. This seriously limits the usefulness of such indices.

### 2.2.2 Bromodeoxyuridine Labelling

As an alternative to thymidine labelling, non-radioactive markers have been developed. Immunohistochemical or flow cytometric (FCM) detection of BrdUrd (or other halagonated pyrimidines) incorporated into DNA either in vivo or in vitro (WYNFORD-THOMAS and WILLIAMS 1986), made possible by the introduction of a monoclonal antibody which recognises BrdUrd (GRATZNER 1982), has revolutionised the study of cell kinetics. BROWMAN et al. (1991) showed a dose response in uptake of BrdUrd when biopsies from oral carcinomas were incubated in vitro, with a mean BrdUrd index rising from 1.6 (±0.05) to 8.8 (±0.9), with a 50-fold rise in incubating concentration (2–100 $\mu M$). Thus optimal conditions for in vitro incubation must be determined before using the technique widely. Many laboratories incorporate BrdUrd under high oxygen tensions (BRITTO et al. 1992) to improve diffusion of the label (Fig. 2). BrdUrd and iododeoxyuridine (IrdUrd) are cytotoxic drugs and may be used in vivo in patients with an established malignancy in a single, low dose (200 mg in 20 ml normal saline) via the intravenous route (WILSON 1991). Although little toxicity has been reported in animal studies, use in humans should be undertaken with caution and certainly with ethical approval. In vivo double labelling using BrdUrd and IrdUrd – both approved for clinical use – allows estimation of potential doubling times of solid tumours (POLLACK et al. 1995).

## 2.3 Immunohistochemical Methods

The principle underlying assessment of cell proliferation by immunohistochemical methods is that there are cell cycle-associated alterations in the amount or distribution of cellular proteins that are recognised as antigens (HALL and WOODS 1990). A wide range of molecules are potential targets for immunohistochemical

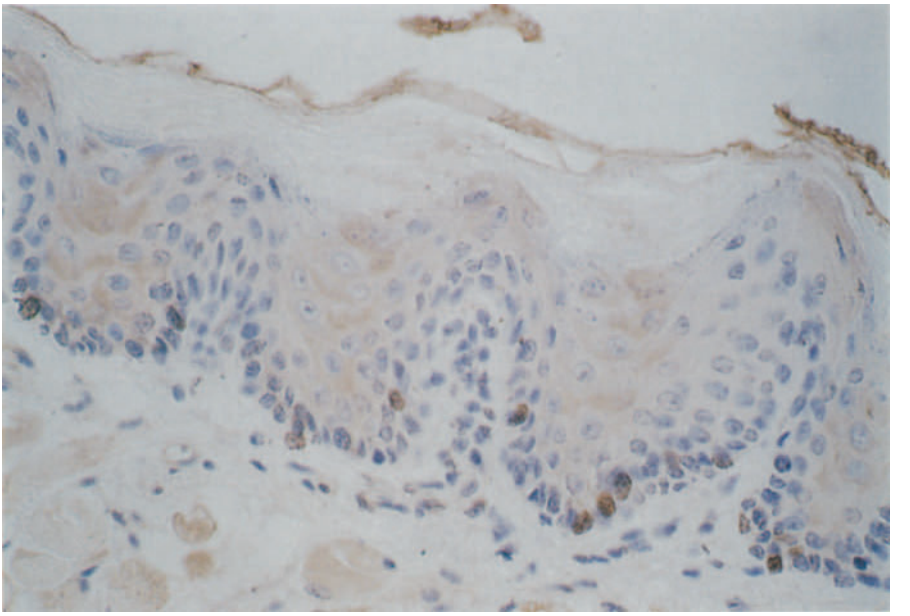


**Fig. 2.** Dorsal mucosa of hamster tongue labelled in vitro with bromodeoxyuridine. There is uniform incorporation of the label in the progenitor compartment across the full width of specimen. ×180

assessment. Here we will focus on two well-characterised antigens, Ki-67 and proliferating cell nuclear antigen (PCNA).

### 2.3.1 Ki-67

Ki-67 antigen is expressed in all phases of the cell cycle except $G_0$ and early $G_1$ (Gerdes et al. 1984), and staining with Ki-67 antibody is widely used as an operational marker of cell proliferation (Yu et al. 1992). Although the half-life of detectable antigen is recognised to be 1 h or less, and Ki-67 expression correlates with other cell proliferation indices, it has been said that it may overestimate the growth fraction (Scott et al. 1991). The first widely available monoclonal antibody against Ki-67 was reactive only on frozen sections (Fig. 3), but genetically engineered MIB-1 and MIB-3 antibodies and polyclonal Ki-67 antibodies can now be used on archival tissues following microwave antigen retrieval (Catterotti et al. 1992). It has been recently shown, by counting MIB-1-stained cells in breast cancer, that some judgement is needed in selecting only moderately-heavily stained MIB-1-positive cells if comparable labelling indices are to be obtained on formalin-fixed, wax-embedded versus frozen tissues (Gee et al. 1995).

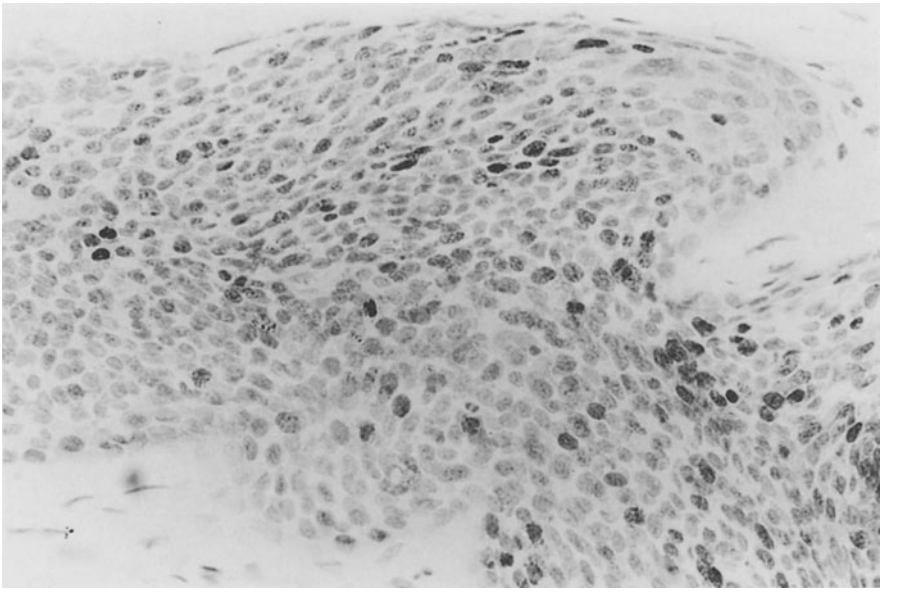


**Fig. 3.** Ki-67 labelling of a frozen section from oral squamous cell carcinoma (SCC) showing strong nuclear labelling of proliferating cells. ×180

### 2.3.2 Proliferating Cell Nuclear Antigen

PCNA is a 36-kDa nuclear protein associated with the cell cycle. A monoclonal antibody that has been generated to genetically engineered PCNA (Waseem and Lane 1990), designated PC10, demonstrates the proliferative compartment (Fig. 4) in conventionally fixed and processed tissues (Hall et al. 1990). The time of fixation affects PCNA immunostaining; good results are observed after 24–36 h, while more than a 48-h fixation is associated with significant reduction in staining intensity (Hall et al. 1990; Lörz et al. 1994). In optimally fixed tissues, positive staining is predominantly nuclear, but cytoplasmic staining is occasionally observed in cells with mitotic nuclei. A gradation of staining intensity is seen from basal to suprabasal cells in stratified epithelia, and criteria for differentiating darkly labelled cells from lightly labelled cells need to be defined. Welsgerber et al. (1993), by enumerating strongly stained PCNA cells using a strictly standardised enumeration method, have shown the PCNA index to be comparable to the BrdUrd index. On the other hand, if all positively stained cells are counted irrespective of the intensity, the PC10 labelling index could be almost fourfold that derived by [$^3$H]dT or BrdUrd labelling (Warnakulasuriya and Johnson 1993a). This poor correlation with other indices has been confirmed in many studies (for a review, see Yu et al. 1992) and may reflect the long half-life of PCNA (about 20 h), resulting in the labelling of some cells that have actually left the cell cycle recently (Hall et al. 1990). Furthermore, very small levels of PCNA may

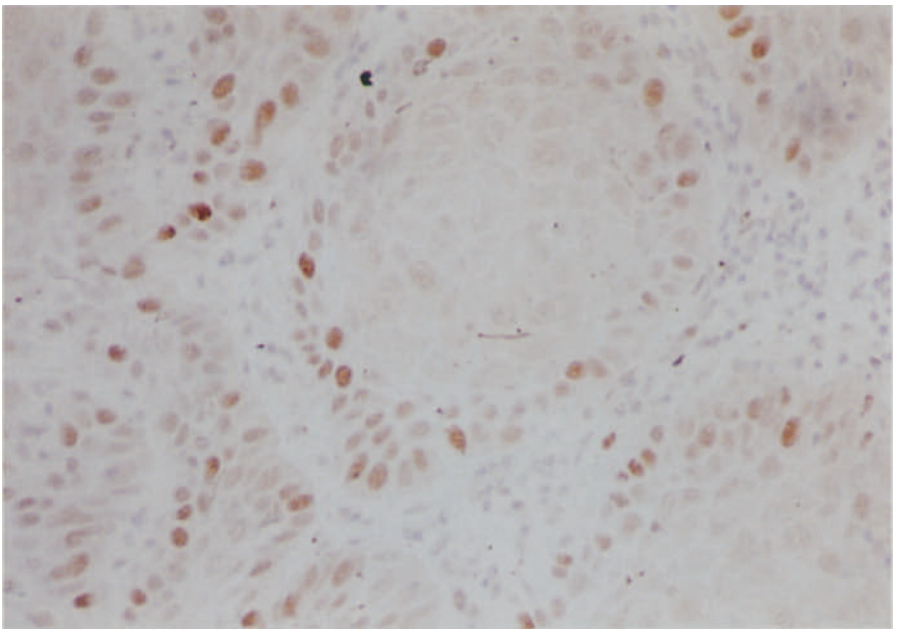


**Fig. 4.** Islands of well-differentiated oral squamous cell carcinoma (SCC) stained against proliferating cell nuclear antigen (PCNA). PC10 immunoreactivity is more frequent in basal cell populations and nearly absent in the central, more differentiated areas. ×180

be found in non-cycling cells with up-regulation occurring on entry to the cell cycle (Bravo and MacDonald-Bravo 1985). Authors using mouse monoclonal antibody 19A2 against PCNA in methanol-fixed tissues claim this immune label is specific for marking S-phase cells (Galand and Degraef 1989; Berlingin et al. 1992).

## 2.4 Argyrophilic Nucleolar Organisation Region Protein Enumeration

The location of nucleolar organisation regions (NOR) is easily demonstrable by the silver-staining method described by Ploton et al. (1986). The argyrophilic NOR proteins (AgNOR) appear as small, dark, intranuclear dots (Fig. 5) in routine histological sections treated with a colloid composed of gelatin and silver formate at room temperature. Their number (per nucleus) has been shown to be correlated with the rate of ribosomal RNA transcription, cell proliferation and DNA ploidy. While the counting of AgNOR is tedious (Crocker et al. 1989), an increased number has been documented in human malignant neoplasms in many tissues (Egan and Crocker 1992). A higher frequency and scattered distribution of AgNOR are hallmarks of increased cell proliferation (Underwood and Giri 1988). For the estimate to be reliable – as with all quantitative assessments – a sufficient and representative number of nuclei have to be counted; Ruschoff et al.

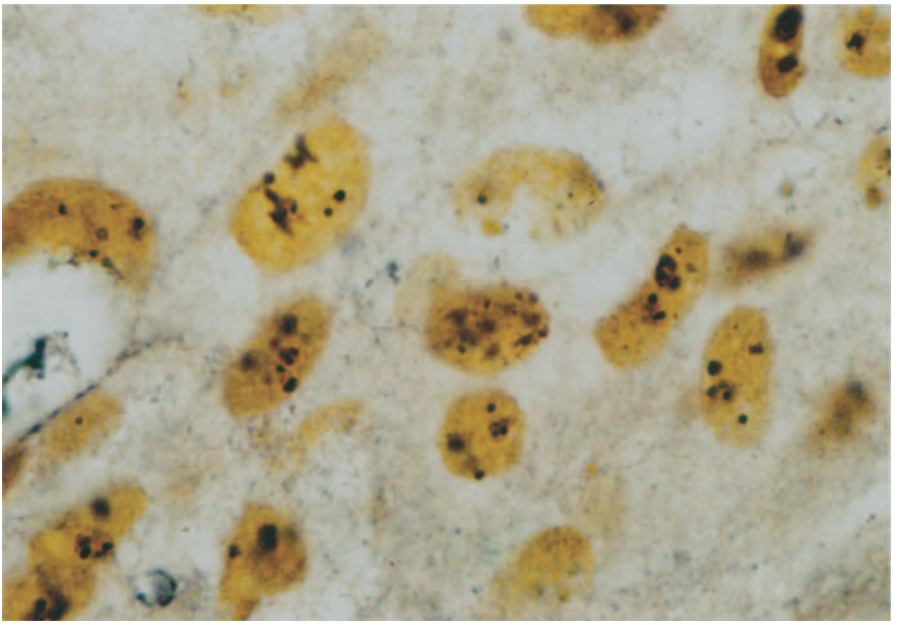


**Fig. 5.** A squamous cell carcinoma with a high number of silver-stained agyrophilic nucleolar organisation regions (AgNOR) per nucleus. AgNOR are widely dispersed throughout the nuclei. ×3580

(1990) recommend the standardised continuous (or running) mean method to arrive at an optimal nuclear count, rather than arbitrarily deciding to estimate 100 or 200 nuclei, as is done in most studies.

## 2.5 Flow Cytometry

DNA analysis by FCM is emerging as an important clinical tool in cell prliferation. In DNA analysis, the fluorochrome most commonly used is propidium iodide (PI). PI binds stoichiometrically to the DNA of cells, and under laser excitation the intensity with which a cell nucleus emits light is directly proportional to its DNA content. Cancer cells often have an abnormal chromosome number that is not a multiple of 23. These aneuploid cells thus emit an abnormal fluorescent intensity. FCM techniques readily divide neoplasms into two categories: diploid cells and aneuploid cells. Broad chromosome numbers, e.g. tetraploidy or other hyperdiploidy, can be recorded with reasonable accuracy. This information can be obtained from single cell suspensions prepared from fresh tissue or thick sections (30–50 $\mu$m) from routinely prepared paraffin-embedded material (Hedley et al. 1983). Using currently available fluorescence-activated cell sorter (FACScan) FCM machines and accompanying computer programmes, about 10000 cells per sample

can be analysed rapidly. However, physical disruption of tissues is required, and results are influenced by the dilution effects of non-tumour/stromal cells. Microdissection of the lesional area obviously improves the specificity of the results. Cytokeratin labels in dual parameter analyses further improve the specificity by recording keratinocytes separately from stromal or inflammatory cells (NYLANDER et al. 1994b).

Several kinetic parameters, i.e. DNA index (DI), denoting the ratio of DNA fluorescence of abnormal to normal $G_1/G_0$ cell populations, S-phase and potential doubling time ($t_{pot}$), a measure of the theoretical poliferative capability of a tumour cell population in the absence of cell loss (STEEL 1977), can be obtained by this method. The technique to estimate $t_{pot}$ is based on the procedure first described by BEGG et al. (1985), whereby a biopsy is taken several hours after incorporation of a label. This method has been used widely to estimate cell proliferation of oral and head and neck tumours in the CRC Gray Laboratory in the United Kingdom (WILSON 1991), and these results are discussed below. With proper sampling time, $t_{pot}$ assessment by FCM is reported to be precise and reproducible (HOYER et al. 1994). The principle advantage of the FCM technique is that it is rapid and the results are available within a day of surgery, allowing the clinician to utilise the information derived in treatment planning.

Using FCM, DNA parameters can also be recorded in parallel with other markers, such as the expression of oncogene (e.g. c-*myc*) or tumour suppressor gene (e.g. p53) proteins, growth factor receptors (e.g. epidermal growth factor receptor, EGFR) or growth factors themselves (e.g. transforming growth factor, TGF; tumour necrosis factor, TNF). These markers are discussed further in Sect. 5.

## 3 Comparison of Methods Used in the Study of Cell Proliferation

Whilst the methods for estimation of cell proliferation described above attempt to measure several different parameters, they are closely interrelated in biological terms. Furthermore, different techniques aimed at measuring the same parameters do not produce strictly comparable results. Nevertheless, kinetic information derived from different approaches using [$^3$H]dT LI, BrdUrd LI and Ki-67 LI were shown to be more or less concordant in non-Hodgkin's lymphomas (SILVESTRINI et al. 1988) and in several other human and mouse neoplasms (WILSON et al. 1985). A more detailed investigation in our laboratory (WARNAKULASURIYA and JOHNSON 1993a) using hamster tongue showed that proliferative indices of simple, flat lining mucosae such as ventral tongue derived by in vivo and in vitro labelling using [$^3$H]dT or BrdUrd were very similar. However, dorsal tongue epithelium, which is thicker, has an undulating morphology, and a complex cell renewal pattern gives markedly and significantly different results with the different methods. There are also potential differences between the information on proliferative status derived by PCNA immunostaining and other established cell cycle markers (see Sect. 2.3). This is not surprising, as it has been shown that PCNA (as detected by PC10) is expressed in non-cycling cells (HALL et

al. 1992). The latter authors concluded that PCNA immunoreactivity can occur without cell proliferation in association with neoplasia. Such methodological differences need to be taken into account when comparing data from different studies.

Other practical difficulties also exist. Techniques such as mitotic and AgNOR counting are time-consuming and are prone to introduce inter- and intra-observer variations, particularly if sampling is inadequate. Measurement of the number of mitoses per ten high-power fields, though quick, easy and inexpensive, has attracted criticism (QUINN and WRIGHT 1990) due to lack of reproducibility. Despite these difficulties, careful work has shown that mitotic activity recorded in this way can be a powerful prognostic marker in breast and ovarian carcinomas (BAAK et al. 1985, 1986; COLLAN et al. 1988; HAAPASALO et al. 1989). DNA FCM is an objective method, which can be performed rapidly on archival tissues. Several mathematical models to estimate the S-phase fraction have been described (SCOTT et al. 1992), and a high variability of S-phase values may result by the use of different methods (SILVESTRINI 1994). There is also conflicting evidence on the correlation between S-phase index determined by FCM and by other labelling methods (STANTON et al. 1991; SILVESTRINI 1994; WALKER and CAMPLEJOHN 1988). Much work on standardisation of methods remains to be done before measures of cell proliferation become routine in clinical practice.

## 4 Clinical Applications in Diagnosis and Prognosis of Oral Lesions

It seems evident from the methodologies described in Sect. 2 that most techniques used to study cell proliferation are complex and time-consuming. As a result, such methods primarily remain research tools. Their value and utility in routine clinical practice remains to be determined. In this section, we examine the evidence from reported studies to highlight the importance of these techniques in diagnosis and grading of oral lesions and, where possible, in predicting the outcome or prognosis.

### 4.1 Cancer

#### 4.1.1 Diagnosis and Grading

Mitotic counting is regarded as useful in differentiating certain malignancies from their benign counterparts, e.g. leiomyosarcoma from leiomyoma (ELLIS and WHITEHEAD 1981). In routine practice, histopathologists *grade* the degree of differentiation of squamous cell carcinomas of the oral cavity in terms of features seen in the microscope image, one of the key features being the so-called mitotic "activity" of the tissue. BRYNE et al. (1991) include the number of mitoses per high-power field as one of the five morphological features used to grade oral cancer. Many of the reports which have used such grading systems do not present data on

individual morphological features, giving only the total malignancy grading score. Data on actual mitotic counts are, as a consequence, meagre for oral cavity carcinomas.

Among the range of proliferative markers, LI appears to be the most researched technique for oral lesions. Data from 12 studies reported between 1974 and 1995 are presented in Table 1, the first five using [$^3$H]dT and the remainder the BrdUrd labelling method, in both cases either in vivo or in vitro. A wide range of mean values are reported (LI, 2.6%–22.4%) and, where both oral and head and neck squamous cell carcinomas were analysed, no consistent differences by site are apparent. Studies which used advanced-stage cancers (T3 or T4) report higher LI values (SAKUMA 1980; MOLINARI et al. 1991). The LI is reported not to be correlated with histology of the tumour (degree of differentiation; HEMMER 1990; CHAUVEL et al. 1983; WILSON et al. 1988), but significant increases are noted of the LI distribution with regard to tumour size as well as lymph node involvement (HEMMER 1990; CHAUVEL et al. 1989; SAKUMO 1980). A higher LI is also recorded in aneuploid tumours (BOURHIS et al. 1994a; NYLANDER et al. 1994a). In the latter study, the mean LI of diploid tumours was 8.7%, compared to 15.6% in aneuploid tumours. Two studies by BENNETT et al. (1992) and NYLANDER et al. (1994), respectively, noted marked differences in LI when measured by histology (14.9%, 13.6%) compared to the FCM technique (6.8%, 9.1%) following BrdUrd incorporation in vivo. Comparison of LI values derived from different studies is fraught with danger because of different reference units used in the estimations. Nevertheless LI for oral carcinomas are considerably higher than those reported for both normal human buccal mucosa (2.48% ± 0.47%) following [$^3$H]dT in vitro labelling (WARNAKULASURIYA and MACDONALD 1993) or for oral leukoplakias reported later in this chapter. LI determination has, however, failed to separate keratoacanthoma from cutaneous squamous cell carcinoma (RANDALL et al. 1990).

Several studies utilising immunohistochemical markers of cell cycle antigens to derive LI in oral carcinomas have been reported recently (Table 2). A strong correlation between PCNA and Ki-67 expression ($p < 0.0001$) was reported by JONES et al. (1994) for head and neck carcinomas. These result in much higher indices and probably overestimate the proliferative fraction (Table 1). A wide range of mean values are reported, particularly for the PCNA index. Recent studies have shown, not surprisingly, that the proportion of Ki-67-positive cells increases with histopathological grade (STRÖKEL et al. 1993; STEINBECK et al. 1993; ZOELLER et al. 1994). These immunolabelling methods are now widely accepted, as they obviate the need for either in vivo and in vitro pre-labelling of tissues; however, before they are adopted for routine use, it is necessary to have a better understanding of how the results obtained compare with previously used techniques.

An increased number of AgNOR counts has been documented in many human malignant neoplasms from various sites, compared to their normal tissue of origin or to benign lesions arising therefrom (EGAN and CROCKER 1992). We reported a higher AgNOR count (8.37 ± 6.11 per cell) in oral squamous cell carcinomas compared to epithelial dysplasia (5.61 ± 4.63) or benign keratosis (4.51 ± 2.57) (WARNAKULASURIYA and JOHNSON 1993b). Although these differences were sig-

**Table 1.** Reported labelling index (LI) of oral and other head and neck carcinomas

| Reference | Carcinomas (*n*) | Tool | Method of labelling | Detection | Mean LI (%) | |
|---|---|---|---|---|---|---|
| | | | | | Oral | Head and neck |
| Bresciani et al. 1974 | 5 | [$^3$H]dT | In vivo | Autoradiography | – | 17.6 |
| Sakuma 1980 | – | [$^3$H]dT | ? | Autoradiography | 22.4[a] | – |
| Silvestrini et al. 1984 | 92 | [$^3$H]dT | In vitro | Autoradiography | 11 | – |
| Chauvel et al. 1989 | – | [$^3$H]dT | ? | Autoradiography | 12.9 | 8.0 |
| Molinari et al. 1991 | 35 | [$^3$H]dT | In vitro | Autoradiography | 14.0[a] | – |
| Wilson et al. 1988 | 9 | BrdUrd | In vivo | Histology | 5.7 | 6.3 |
| Hemmer 1990 | 33 | BrdUrd | In vitro | Histology | 2.6[b] | – |
| Bennett et al. 1992 | 123 | BrdUrd | In vivo | Histology | – | 14.9 |
| Wilson et al. 1995 | 99 | BrdUrd | In vivo | Histology | – | 8.1 |
| Forster et al. 1992 | 82 | BrdUrd | In vivo | FCM | – | 8.0 |
| Lochrin et al. 1992 | 38 | BrdUrd | In vivo | FCM | – | 7.1 |
| Bourhis et al. 1994a | 49 | BrdUrd | In vivo | FCM | 8.5[c] | – |
| Nylander et al. 1994a | 31 | BrdUrd | In vivo | FCM | – | 13.6 |
| Jones et al. 1994 | 75 | BrdUrd | In vivo | FCM | – | 8.9 |

[$^3$H]dt, tritiated thymidine; BrdUrd, bromodeoxyuridine; FCM, flow cytometry.
[a] Only advanced stage (T3, T4) tumours were included.
[b] Some cancers recorded 0% LI, leading to a low mean value; the range was 0%–23%.
[c] Included some oropharyngeal tumours.

**Table 2.** Mean labelling indices (LI) for oral and head and neck carcinomas derived by immunolabelling techniques

| Reference | Carcinomas (*n*) | Labelling index (%) | |
|---|---|---|---|
| | | Ki-67[b] | PCNA[c] |
| KEARSLEY et al. 1990 | 42 | 2–52 | – |
| TSUJI et al. 1992 | 48 | – | 11.6 |
| GÜNZL et al. 1993 | 40 | – | 3.9–7.6 |
| STÖRKEL et al. 1993[a] | 100 | – | 40–78 |
| WARNAKULASURIYA et al. 1994 | 20 | 27.1 | 67.4 |
| ROLAND et al. 1994 | 79 | 27.8 | – |
| GIROD et al. 1994 | 144 | – | 29–50 |
| LÖRZ et al. 1994 | 53 | – | 17.5 |
| JONES et al. 1994 | 75 | 29.8 | 56.0 |
| TSAI and JIN 1995 | 38 | – | 29.2 |

PCNA, proliferating cell nuclear antigen.
[a]Lower mean LI (40%) in grade 1 and higher value (78%) in grade 3 (Bryne's malignancy grading).
[b]For Ki-67 LI monoclonal antibody against Ki-67 in frozen sections.
[c]For PCNA, most studies report use of PC10 antibody.

nificant, counts in each diagnostic group overlapped so much that they were of no practical value in distinguishing between individual lesions. However, the higher counts found in many carcinomas were due to dispersion of AgNOR within the nucleoplasm (Fig. 5), so that AgNOR *type* is more useful in making a diagnosis of malignancy than the actual *count*. Several other laboratories have confirmed these results (CHUNGPANICH and SMITH 1989; PICH et al. 1992; CHATTOPADHYAY et al. 1994).

Many studies on oral squamous cell carcinomas have reported on the measurement of DNA content (ploidy status) by FCM. The biological rationale behind the measurement of nuclear ploidy is that deviation from normal nuclear complement of DNA is likely to reflect cellular aberration and resistance to growth control (MILLER 1992). The ploidy status of oral and head and neck carcinomas has been examined rather extensively (Table 3). Close to 50% of oral and head and neck cancers have aneuploid DNA patterns. A higher percentage of aneuploid tumours are noted in oral cavity and tongue cancers compared to lip cancers (STEINBECK et al. 1993). Aneuploidy appears to be associated with less well differentiated tumours (TYTOR et al. 1987; SUZUKI et al. 1994) and more advanced clinical stages, including lymph node metastasis (TYTOR et al. 1987; BALSARA et al. 1994; SUZUKI et al. 1994).

Examining the ploidy status, CHEN et al. (1993) confirmed that the incidence of lymph node metastasis in the aneuploid tumours (70%) was significantly higher than for diploid tumours (24%). A comparison of the ploidy status of primary and metastatic squamous cell carcinomas (SUZUKI et al. 1994) revealed that most metastatic lymph nodes were in fact diploid. This may suggest that a primary neoplasm may have increased incidence of aneuploidy with the increase in size of the tumour, but that diploid cells rather than aneuploid cells are responsible for causing lymph node metastasis.

**Table 3.** Ploidy states and potential doubling time ($t_{pot}$) in oral and head and neck carcinomas by flow cytometry (FCM)

| Reference | Carcinomas (*n*) | Aneuploid (%) | $t_{pot}$ (days) |
|---|---|---|---|
| WILSON et al. 1988 | 9 | – | 6.2 ± 2.7[b] |
| FORSTER et al. 1992 | 82 | 63 | 6.2 |
| NYLANDER et al. 1994 | 31 | 65 | 4.6 |
| VAN HEERDEN et al. 1995 | 50 | 44 | – |
| SICKLE-SANTANELLO et al. 1986 | 15 | 66 | – |
| TYTOR et al. 1987 | 88 | 48 | – |
| GOLDSMITH et al. 1987 | 48 | 48 | – |
| BENNETT et al. 1992 | 123 | 41 | 1.8–41.2 |
| LOCHRIN et al. 1992 | 38 | – | 3.9 |
| BOURHIS et al. 1994a | 49 | 59 | 5.07 |
| WILSON et al. 1995 | 99 | 49 | 3.9 |
| SUZUKI et al. 1994 | 41 | 39 | – |
| BALSARA et al. 1994 | 68 | 42[a] | – |
| BENAZZO et al. 1995 | 52 | 20 | 5.7 |

[a] Includes tetraploid tumours.
[b] Estimated from author's own data.

Recent FCM studies also attempt to estimate the potential doubling times ($t_{pot}$). Mean $t_{pot}$ values recorded by FCM in six studies on head and neck carcinomas range from 4 to 6 days (Table 3), but a wide range is reported for individual tumours: 1.8–41.2 days by BENNETT et al. (1992) and 1.3–12.2 days by NYLANDER et al. (1994a). Aneuploid tumours have shorter median $t_{pot}$ values and higher LI indices than diploid tumours (BOURHIS et al. 1994a; NYLANDER et al. 1994a). Proposals for combining these three variables in prognostication is discussed in Sect. 4.1.2.

### 4.1.2 Prognosis

At present most institutions use a range of static descriptors of the primary neoplasm (JONES 1994; BRYNE et al. 1991), as well as a range of host factors, in determining the prognosis and treatment plan for patients with oral carcinoma (JOHNSON et al. 1996). However, in order to obtain a better approximation of biological potential, the morphological grade should be supplemented by functional attributes, such as proliferation markers. Contrary to this observation, JOHNSON (1976), employing a multifactorial analysis based on 39 different histological features on 100 cases of oral carcinomas, found many of the variables, including the degree of mitotic activity, had little or no prognostic value. The reason for expecting that features of cellular proliferation will be useful in prognosis is that they reflect the rate of tumour growth. The faster the velocity of the tumour, the quicker it will spread (EVANS et al. 1982).

LI derived from [$^3$H]dT/BrdUrd or from immune labelling have value in predicting the short-term clinical response to radiotherapy (SILVESTRINI et al. 1984; GÜNZL et al. 1993) and chemotherapy (MOLINARI et al. 1991); this is not surpris-

ing, as rapidly dividing cells are more vulnerable to the effects of ionising radiation or cytotoxic agents. BETTINGER et al. (1991) and BOURHIS et al. (1994b) used kinetic methods to assess success or failure of induction chemotherapy in head and neck squamous cell carcinomas. Rapid cell proliferation was noted among patients who had responded poorly to therapy. Immune markers have not been examined in large-scale retrospective or prospective studies to determine their value in relation to recurrence rates or survival times. Two recent studies of PC10 and Ki-67 indices did not find the data useful as predictors of lymph node metastasis or of survival (JONES et al. 1994; ROLAND et al. 1994). Contradicting results were reported by STÖRKEL et al. (1993) and GIROD et al. (1995). SANO et al. (1991), who examined the prognostic implication of AgNOR enumeration in 39 patients with oral carcinoma, showed that the 5-year survival rate of patients with high AgNOR (>6.5) was 38% and significantly lower than the group with an AgNOR count of less than 6.5 (5-year survival rate, 67%). PICH et al. (1992) also claim that a lower mean AgNOR count per nucleus may reflect an improved survival.

KEARSLEY and THOMAS (1993), examining a range of potential proliferation markers, showed ploidy status to be of value in head and neck cancer; their review suggests that patients with aneuploid tumours had substantially worse prognosis. Aneuploid tumours are also known to have a shorter $t_{pot}$ than diploid tumours (FORSTER et al. 1992) and, as observed above, because $t_{pot}$ measures the velocity of tumour growth, it is likely to prove valuable in predicting both relapse and survival. A complete response reported for many tumours with a long $t_{pot}$ (>7.0 days) by NYLANDER et al. (1994) provides some promise for future studies. Many small retrospective studies have yielded inconsistent results, and larger prospective studies are needed to validate the proliferation markers developed so far. A subset of oral squamous cell carcinomas characterised by an LI greater than 15%, , DNA aneuploidy and a $t_{pot}$ of less than 5 days may benefit from more aggressive therapies, such as accelerated regimes of radiotherapy and/or other multimodal therapies compared to slow-growing tumours (LI, <15%; $t_{pot}$, >5 days) showing DNA diploidy. Further multicentric studies are needed to test kinetic parameters which – either alone or in combination – may provide useful and accurate prognostic information. The cell kinetics data used for treatment planning should also be subjected to external quality assessment in one central laboratory.

It is also worth reiterating that a true understanding requires concomitant measures of apoptosis and of a large number of host response variables, together with absence or continuous presence of aetiological agents, notably tobacco and alcohol use.

## 4.2 Potentially Malignant Lesions

### 4.2.1 Leukoplakia

There is considerable interest in the characterisation of cellular and molecular markers that may actually predict which potentially malignant lesions of the oral

cavity may, with time, transform to cancer (BURKHARDT 1985; JOHNSON et al. 1993, 1996). Although the majority of proliferating cells in a keratinising epithelium may contribute little, if any, to the process of malignant transformation in vivo (HUME 1981) most, but not all, "precancerous" lesions and conditions are associated with periods of increased cell proliferation (IVERSON 1992). Some of the histological features commonly recognised as components of oral epithelial dysplasia (SMITH and PINDBORG 1969) relate to the proliferation state of the tissue. Mitotic index is reported to be higher in parakeratotic than in orthokeratotic oral lesions (RENSTRUP 1963; MAIN 1965; EL-LABBAN et al. 1971); the former metaplastic variety of keratosis is more often associated with malignant potential, suggesting that increased cell proliferation may indirectly have a role in transformation to cancer. Kramer's group, in a discriminant analysis of histological features of oral dysplasia, recognised three factors as important determinants of subsequent malignant transformation (KRAMER et al. 1970): (1) presence of abnormal mitoses, (2) increased mitotic activity and (3) mitosis in superficial layers of the epithelium (Fig. 1). Regrettably, long-term, follow-up studies of oral leukoplakia concerning "histopathological risk markers" are scarce.

During the 1970s, a few studies attempted to determine the LI of oral leukoplakia by in vitro incorporation of [$^3$H]dT. Available data on the cell proliferation status of leukoplakias compared to control tissues are shown in Table 4. Although the criteria used for the selection of leukoplakias and the methods of determining reference indices are likely to be different in these various studies, there is an interesting small, but significant rise of LI in leukoplakia specimens compared to normal tissues. A shift in labelling of proliferative cells from the suprabasal to the basal compartment was reported in leukoplakias by MAIDHOF and HORNSTEIN (1979). This was confirmed by WARNAKULASURIYA and MACDONALD (1995b), who showed that the rise in LI was related to an increase in the proportion of the cells in the basal cell layer engaged in cell production. In one of these studies (WARNAKULASURIYA 1976), in vitro double labelling showed that the time spent by cells in S phase ($t_s$) is not significantly altered in leukoplakia. The LI itself was, therefore, regarded as a sufficient indicator of cell proliferation. The observation by WARNAKULASURIYA and MACDONALD (1995b) that the ranking of LI in leukoplakia specimens correlated ($r$, 0.50; $p = 0.019$) with the severity of epithelial dysplasia scored by the method of SMITH and PINDBORG (1969) lends support to the importance of individual features classically assumed to indicate

**Table 4.** Labelling indices of oral leukoplakia and normal mucosa

| Reference | Reference index | Labelling index (%) | | Lesion to control ratio | $p$ value |
|---|---|---|---|---|---|
| | | Leukoplakia | Controls | | |
| ALVARES et al. 1972 | LC/TNC | 4.75 | 2.72 | 1.75 | <0.01 |
| WARNAKULASURIYA 1976 | LC/TNC | 6.01 | 3.39 | 1.77 | <0.005 |
| MAIDHOF and HORNSTEIN 1979 | LC/BC | 6.62 | 5.53 | 1.20 | <0.01 |
| CRISCUOLO et al. 1989 | LC/TNC | 5.3 | 2.6 | 2.0 | <0.004 |

LC/TNC, labelled cells per 100 total nucleated cells; LC/BC, labelled cells per 100 basal cells.

proliferation state in histology grading systems. Conversely, LI as determined by [$^3$H]dT may prove to be an important objective criterion to assess the probability/risk of transformation to cancer.

Steinbeck et al. (1993) observed that PCNA immunoreactivity in basal cell layers of oral precancerous lesions increased with increasing grade of dysplasia. Mean PCNA LI values recorded in their study were as follows: normal, 17%; mild, 49%; moderate, 54%; and severe dysplastia, 73%. With increase in severity of dysplasia grade, PCNA-immunoreactive cells increased in number in the basal cell layer. In severe dysplasia, however, PCNA-positive cells were abundant in superficial cell layers. No follow-up information was given on these oral lesions, so the predictive value of the kinetic indices remain unknown. Cell proliferation detected by PCNA LI is also reported to be increased in *Candida*-associated leukoplakia (Warnakulasuriya et al. 1994). The mean PC10 count in this study for the *Candida* group was 33.8% ± 12.2% compared to 23.6% ± 14.4% in control leukoplakia samples ($p = 0.057$). Whether this rise in LI in the *Candida*-associated lesions was caused by factors released by the organisms or cytokines from the immune/inflammatory responses is unknown, but both are certain to be involved, directly or indirectly.

AgNOR enumeration, commonly used for neoplastic discrimination (see Sect. 4.1.1), has also been applied to oral precancer by several groups. Leukoplakia lesions with histologically defined dysplasia have been shown to have a higher mean AgNOR count per nucleus compared to normal mucosa or simple keratoses (Warnakulasuriya and Johnson 1993b; Chattopadhyay et al. 1994). The range of the scatter of the AgNOR values is, however, too wide for this information to be of any potential value in detecting oral leukoplakia with poor prognosis. Rajendran and Nair (1992), applying the AgNOR technique to oral submucous fibrosis (OSF) cases from India, reported a two- to threefold increase of AgNOR numbers in the OSF epithelium compared to normal mucosa. This is surprising, since moderate and advanced OSF cases included in their study demonstrate marked epithelial atrophy compared to normal controls.

DNPA ploidy status of oral leukoplakia has been examined by FCM to a limited degree. Grässel-Pietrusky et al. (1982), Saito et al. (1991) and Kahn et al. (1992), utilising small sample sizes, described aneuploidy in eight out of 24, two out of 11 and six out of 19 leukoplakia lesions, respectively. All these studies provide examples of aneuploidy being more frequently observed in severely dysplastic lesions than in simple keratoses. Further studies are needed to clarify whether determination of ploidy status has a value in diagnosis of more serious lesions and in predicting likelihood of malignant transformation.

### 4.2.2 Lichen Planus

Malignant development in oral lichen planus is reported. This, however, is a very rare event (see D.M. Williams, this volume) but full assessment of subjects at risk is of considerable value in clinical practice. Oral lesions with "lichenoid dysplasia" have been reported, but follow-up information on these is very meagre. Few attempts have been made to characterise cell kinetics of oral lichen planus. Walker and Dolby (1974), examining the in vitro [$^3$H]dT labelling indices of 17

lichen planus lesions compared to normal controls ($n = 10$), reported a significant rise in basal LI in both atrophic and acanthotic lesions. No differences were found when the LI was plotted by the total cell count of the epithelium. A more recent study of oral lichen planus has confirmed this shift of proliferation to the basal compartment (SARDELLA et al. 1991). This may reflect the loss or liquefaction of non-dividing basal cells. Basal cell proliferation, on the other hand, may be induced by cytokines derived from the immune inflammatory response at and below the basement membrane. More work is needed to understand the implications of these preliminary findings.

## 4.3 Salivary Neoplasms

Malignant salivary gland neoplasms are rare, consitute a heterogenous group and are often difficult to diagnose histologically. Immunohistochemical methods that are useful in diagnosis of malignant salivary gland tumours were reviewed by SEIFERT (1992). AgNOR enumeration in salivary neoplasms has been reported by many authors to be of potential diagnostic value (Table 5). The differences between mean NOR counts in malignant salivary neoplasms (×4.5) and benign adenomas (×1.5) is about two- to threefold in most studies (KAMATH and SASTRY 1994). LANDINI (1990), examining NOR in pleomorphic adenomas, reported that cells in solid/ductal areas had a higher NOR count than chrondroid cells. Application of AgNOR enumeration to examine its potential value in determining prognosis of muco-epidermoid carcinoma was described by CHOMETTE et al. (1991). AgNOR in 15 cases with poor prognosis (fatal outcome or early recurrence and/or metastasis) were 2.8–6.2 per nucleus compared to 1.0–3.6 per nucleus in a group with good prognosis (no recurrence in 5 years). The AgNOR count seemed better than histological criteria for establishing the prognosis of muco-epidermoid tumours.

Application of other methods to investigate cell proliferation has been meagre. Poliferative activity of pleomorphic adenomas and myo-epitheliomas were compared by PCNA labelling and no significant differences were reported, suggesting that the method has no potential value in discrimination (OGAWA et al. 1993). Not surprisingly, marked differences in PCNA index were shown between carcinomas in expleomorphic adenomas ($22.9 \pm 6.2$) and pleomorphic adenoma ($6.9 \pm 3.4$) by YANG et al. (1994). Using Ki-67 antibody on six malignant salivary tumours, MURAKAMI et al. (1992) demonstrated a high proliferation status (18.3% cells positive compared to 4.7% in normal salivary gland tissue).

An FCM study on a series of muco-epidermoid, acinic and adenoid cystic carcinomas revealed very few aneuploid DNA stem-lines; when present, this feature was seen largely in undifferentiated carcinomas. The survival time of these patients with aneuploid tumours was considerably reduced compared to those with diploid tumours (BANG et al. 1994).

A cytophotometric analysis of DNA to assess the aggressiveness of adenoid cystic carcinomas of salivary and lacrimal glands revealed that the method is useful to define the ploidy status. Tumours with diploid histograms had the longest survival, and those with aneuploidy had the worst prognosis (HAMPER et al. 1990).

**Table 5.** Mean argyrophilic nucleolar organisation region (AgNOR) counts per nucleus in salivary neoplasms

| Histology | Mean AgNOR values | | | | | |
|---|---|---|---|---|---|---|
| | Morgan et al. (1988) | Van Heerden and Raubenheima (1991) | Matsumura et al. (1989) | Cardillo (1991) | Chomette et al. (1991)[a] | Landini (1990)[b] |
| Pleomorphic adenoma | 1.47 | 1.52 | 1.62–1.68 | 1.67 | – | 1.30–2.06 |
| Clear cell adenoma | – | – | 1.47 | – | – | – |
| Warthin's tumour | – | – | 1.72 | – | – | – |
| Adenocystic carcinoma | 3.93 | 2.83 | 2.78 | 3.38 | – | – |
| Adenocarcinoma | – | – | 2.25 | 2.07 | – | – |
| Mucoepidermoid carcinoma | 4.25 | 1.93 | 2.59 | – | 1.0–3.6<br>2.8–6.2 | – |
| Squamous cell carcinoma | – | – | – | 4.31 | – | – |
| Carcinoma (originally pleomorphic adenoma) | – | – | – | 4.87 | – | 6.05 |

[a] 16 cases with poor prognosis and 15 cases with good prognosis.
[b] Range shown is for chondroid versus solid/ductal areas in pleomorphic adenoma.

### 4.4 Jaw Lesions

Following Toller's original description of high rates of epithelial proliferation in the lining of odontogenic keratocysts (Toller 1971), immunohistochemical markers have recently been applied to this issue. Using immunohistochemistry, Li et al. (1994) demonstrated that PCNA-positive cells were mostly located in the suprabasal layers of odontogenic keratocysts (OKC), with less than 5% labelled cells distributed in the basal layers. OKC also had a much higher LI (in some instances of the order of ten- to 20-fold) compared to radicular or dentigerous cysts. Greater proliferative activity of OKC is in accord with their aggressive clinical behaviour. Subsequent studies by the same group using Ki-67 antibody showed that LI in simple and recurrent OKC were not different. OKC associated with basal cell naevus syndrome, however, had a markedly elevated Ki-67 index. Jaw cysts associated with the syndrome had a LI which was nearly twice that of single or recurrent OKC. The Ki-67 method does not appear to allow detection of a subgroup of OKC which are more likely to recur, and it was concluded that recurrence following surgery may be linked to incomplete excision rather than any differences attributable to intrinsic biological behaviour of such cysts. Significant differences in proliferative indices in different cyst types have now been confirmed by other studies (Lombardi and Morgan 1994; Slootweg 1995). Comparing the published data on odontogenic cyst linings (Li et al. 1994) with PCNA LI derived for unicystic ameloblastoma, it was shown that the latter group contained significantly more PCNA cells (Li et al. 1995b). This study also demonstrated significant differences in the proportions of labelled cells between unicystic and solid ameloblastomas and between unicystic lesions with and without invading tumour islands. These differences in proliferative capacity may explain the differences in behaviour between ameloblastomas that are basically cystic and those which, in addition, contain tumour islands invading the fibrous wall and which are known to have a greater potential to recur.

The other pathological entity among jaw lesions which has received some attention is the giant cell lesion. Whitaker et al. (1993) applied AgNOR techniques to differentiate aggressive/recurrent lesions and reported significant differences in mean AgNOR counts in both mononuclear and multinucleate cell populations of clinically aggressive/recurrent compared to non-aggressive lesions. Close examination of these data shows that most of the mean AgNOR counts derived fall between one and two dots per nucleus for all groups of lesions and are therefore unlikely to be of practical diagnostic value.

## 5 Interaction of Cell Proliferation Assessment with Oncogenes and Tumour Suppressor Genes that Control the Cell Cycle

The relatively uncontrolled proliferation that characterises neoplastic cell populations is now recognised to result from specific gene mutations, amplifications or losses. It has recently been suggested that *all* human neoplasms have aberrations

of one or more cell cycle genes (Clurman and Roberts 1995). Activation of several proto-oncogenes and the overproduction of growth factors and their receptors (e.g. *ras*, PRAD-1, *c-myc*, EGF/c-*erbB*) may result in persistent mitogenic signalling or, conversely, loss of tumour suppressor genes (e.g. *p53*, *Rb*) may result in a cell becoming less responsive to negative growth factors. The genes which control mitosis and apoptosis in oral squamous cell carcinoma were reviewed by Scully (1993), Sugerman et al. (1995) and Johnson et al. (1996). Lack of control of these molecular mechanisms are the fundamental stigmata of the malignant phenotype. In this section, we briefly review the current knowledge on associations of proliferation markers and oncogene/tumour suppressor gene alterations in oral and salivary neoplasms.

It has been proposed that the autocrine production of growth factors is an essential element of tumourigenesis (Pusztai et al. 1993). EGFR is a transmembrane glycoprotein (Fig. 6) whose expression is important in the growth regulation of many neoplasms, particularly in breast cancer, where in some studies it is suggested to be a significant and independent indicator of a likely recurrence (Gasparini et al. 1992). Strong EGFR expression in oral squamous cell carcinoma has been associated with a comparatively short survival time (Störkel et al. 1993), and this group recommend it as a valuable tool in estimating the patient's prognosis. The amplification of an associated proto-oncogene, c-*erbB*, has been linked to carcinogenesis in the hamster cheek pouch, with the amplified product being expressed at the time of early invasion and increasing in parallel with the increased

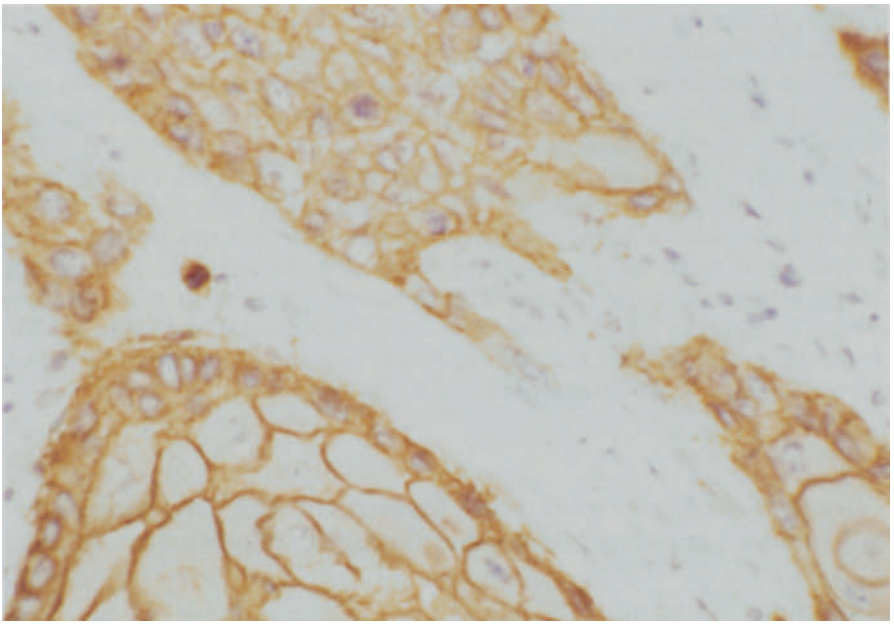


**Fig. 6.** Epidermal growth factor receptor (EGFR) staining in oral squamous cell carcinoma (SCC). The immunoreactivity is strong and mostly membranous. ×990

tumour burden (WONG and BISWAS 1987). The c-erb2 oncoprotein is known to be infrequently expressed in salivary gland tumours (KERNOHAN et al. 1991) and was reported to be confined to malignant cells of carcinoma of expleomorphic adenoma (SHRESTHA et al. 1992). When present, c-*erb2* amplification may be associated with more aggressive tumour behaviour (BIREK et al. 1994).

The $G_1$ to S phase and also $G_2$ to M transitions require prior synthesis and accumulation of cyclins to critical threshold levels. There are at least eight distinct cyclin genes in the mammalian genome (HUNTER 1993). Of these, the cyclin D1 (PRAD-1) gene is shown consistently to be amplified in many human tumours (HINDS et al. 1994). Overexpression of D-type cyclins was recently described in 25 out of 52 human head and neck carcinomas (48%; BARTKOVA et al. 1995) using a monoclonal antibody (DCS6) on archival specimens. These authors' observations on antibody-mediated neutralisation of cyclin D1 and D2 in five head and neck squamous cell carcinoma cell lines has provided evidence for the cooperative roles played by cyclin D1 and D2 in persistent regulation of $G_1$/S entry. Although there is no proof that this effect, when stimulated, is causal in squamous cell carcinoma, comparative analysis of cell cycle markers and D-type cyclin expression may further explain the $G_1$ dysregulation in cancers of the oral/head and neck region. Such dual assessment may, perhaps, allow us to identify a subset of neoplasms that are rapidly and uncontrollably proliferating and, therefore, aggressive in nature.

Expression of c-*myc* is rapidly induced by a variety of growth factors, and this is critical in deciding whether a cell is to grow or die in response to external stimuli (HUNTER 1993; IVAN and LITTLEWOOD 1993). Although it has been shown that c-*myc* alone may drive quiescent cells into the cell cycle, few data exist to prove any variation in the level of c-*myc* in continuously growing cells. In oral squamous cell carcinoma, c-*myc* expression may indicate poor prognosis (FIELD et al. 1989). Nuclear labelling of c-*myc* has been shown to reflect progressive histological changes in pre-cancer and early cancerous lesions of the oral cavity (EVERSOLE and SAPP 1993).

Wild-type p53 is a growth suppressor protein, the expression of which inhibits the growth of both normal and transformed cells. The *p53* checkpoint controls entry to S phase by cells containing damaged DNA. Delaying S-phase entry allows the cell time to repair DNA damage in an extended $G_1$ phase, thereby avoiding propogation of permanent genetic damage. Overexpression of p53 protein (presumptively mutated or otherwise inactivated) has been reported in 11%–80% of oral squamous cell carcinoma. Three recent studies have examined the relationship of overexpression of p53 oncoprotein with the state of cell proliferation in oral carcinoma (WARNAKULASURIYA and JOHNSON 1994; SLOOTWEG et al. 1994; BOURHIS et al. 1994a). Cell proliferation was found to be significantly higher in p53-positive neoplasms. WARNAKULASURIYA and JOHNSON (1994) argue that inactivated *p53* probably confers a growth advantage to the *p53*-positive neoplasms. It has emerged that the p53 protein is a sequence-specific DNA-binding protein that can activate or repress transcription of other genes such as *p16/waf-1* which participate in the cell cycle. Deactivation of *p53* by mutation or deletion may therefore, through complex molecular pathways, result in blocking apoptosis and allow cell proliferation to proceed unabated, a change also necessary for malignant

transformation (Sugerman et al. 1995). Overexpression of p53, now known to be a hallmark of neoplasia and for which detection systems are routine in many laboratories, may itself reflect the cell proliferation status because of its close interaction with cell proliferation/apoptosis balance.

## 6 Conclusions

The methods commonly employed for the assessment of cell proliferation have been critically considered. As interest in the assessment of proliferative activity has grown, so has the number of techniques devised to extract proliferation-related information from processed tissues. The availability of monoclonal and polyclonal antibodies for immunohistochemical detection of antigens related to the cell cycle – particularly Ki-67 and PCNA – have opened up new possibilities with fixed tissues, obviating the need for in vivo and in vitro pulse labelling. Oral pathology laboratories need to utilise these tools, particularly to facilitate staging and grading of oral lesions, so that objective assessments can be introduced in defining lesions at risk.

The pathological assessment, i.e. malignant grading, of oral squamous cell carcinoma has remained unchanged for decades. The measurement of cellular DNA content by FCM is emerging as a prognostic aid in many human tumours. The value of FCM in diagnosis and prognosis of oral cancer needs clarification. Prospective clinical trials are needed to examine the usefulness of combinations of cell kinetic markers (e.g. LI, ploidy and $t_{pot}$) in prognostication and in the choice of the most effective therapy for the individual patient.

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# Suppressor Protein p53 and Its Occurrence in Oral Tumours

P.J. SLOOTWEG



## 1 Introduction

As summarised by LEVINE and MOMAND (1990), a cancer cell is the result of a multistep process in which multiple sequential mutations occur. In this process, two distinct categories can be discerned: the inappropriate activation of proto-oncogenes to become oncogenes and the inactivation of tumour suppressor genes, both of which may lead to disturbed cell proliferation and the development of tumours (CHANG et al. 1993). One of the most intensely studied tumour suppressor genes is the *p53* gene. The protein encoded by this gene appears to act as "the guardian of the genome" (LANE 1994) by blocking the division of cells that have sustained DNA damage, resulting in either a delay in progress through the cell cycle to permit repair or triggering cell death by apoptosis, thus eliminating abnormal cells that could lead to cancer (LEVINE et al. 1994). Moreover, p53 appears to play a role during embryogenesis, as mice lacking the *p53* gene exhibited an increased incidence of neural tube defects (SAH et al. 1995) and an increased sensitivity to an environmental teratogen (NICOL et al. 1995).

This contribution will focus upon the occurrence of p53 alterations in oral tumours and their significance; before this, historical background, more general aspects and the various means currently available for analysis of the *p53* gene will be briefly discussed.

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## 2 Historical Background

The story of the discovery of the p53 protein is connected with the production of the Salk polio vaccine in monkey kidney cells. From a shift from rhesus monkeys to African green monkeys, it became apparent that the extract of cells of the former had a cytopathic effect on the kidney cells of the latter kind of monkey. Subsequent analysis revealed this effect to be due to a virus that initiated cancers in hamsters. This virus was named SV40 (simian vacuolating virus 40). SV40 expressed a viral protein, the large T antigen, which was shown to form a tight complex with a nuclear phosphoprotein, the p53 protein. Subsequent studies revealed that the p53 protein was present in minute amounts in normal cells, but in high levels in tumour cells. Initially, the protein was classified as a tumour antigen and thereafter as an oncogene product but finally, as all p53 protein in neoplastic cells turned out to be the product of a mutated gene, *p53* appeared to represent a tumour suppressor gene (Lane and Benchimol 1990; Levine et al. 1991; Levine 1994).

## 3 Role of *p53* in Tumourigenesis

Evidence that *p53* is a tumour suppressor gene is twofold. Firstly, *p53* mutations in human cancers commonly take the form of a missense mutation in one allele and the loss of the other. This complete loss of both wild-type alleles is typical of a tumour suppressor gene. Secondly, mice with both *p53* alleles mutated develop cancers within 6–9 months, showing that absence of *p53* function predisposes to cancer (Berns 1994; Levine et al. 1994; Picksley and Lane 1994). Mutation of one allele in the germ line results in cancers occurring at unusually early age, a condition known as the Li-Fraumeni syndrome (Birch 1992, 1994).

However, there is some evidence that in some aspects the *p53* gene behaves differently from an ordinary tumour suppressor gene, in which the loss of function is responsible for tumour development. This evidence comes from observations that indicate that the mutant protein may block the function of the wild-type protein if one allele is mutated and one is wild-type *p53*. Moreover, mutant p53 proteins may be actively involved in cell transformation. Therefore, the *p53* gene has features of an oncogene as well as of a tumour suppressor gene. Mutations resulting in loss of function and acquisition of new activities may both be responsible for tumour development (Lane and Benchimol 1990; Levine et al. 1994).

Normally, the p53 protein acts as a checkpoint control in the cell cycle, inhibiting progression of cells in $G_1$ by inducing expression of genes that block the cell cycle (El-Deiry et al. 1993) and preventing entry into S phase in the event of damaged DNA. Moreover, the p53-mediated DNA damage response includes an inducible DNA repair component (Smith et al. 1994). Thus the duplication of damaged DNA is prevented. This normal function of p53 can be abolished in several ways. Firstly, no normal protein is present due to a loss of one *p53* allele together with a missense mutation of the other *p53* allele. Secondly, the product of

a mutated *p53* allele blocks the activity of the wild-type protein encoded by the remaining normal allele. Thirdly, the normal protein is blocked in its activity by binding to another protein; this may be a viral protein from SV40, from human adenovirus or from human papillomavirus (HPV), but binding to a cellular gene product such as the murine double minute-2 (MDM-2) may also lead to inactivation of p53 protein (MOMAND et al. 1992; MELTZER 1994).

## 4 Analysis of *p53* and Its Protein

The *p53* gene encompasses 20 kilobases (kb) of DNA on the short arm of chromosome 17 at position 17p13.1. The product of the gene is a 393-amino acid nuclear phosphoprotein of about 53kDa (hence its name p53 protein) (LEVINE and MOMAND 1990). The gene consists of 11 exons; exons 2–11 code for the p53 protein. There are five regions of the p53 protein in which amino acid sequence in several species is similar over lengths of up to 20 amino acids. These are the so-called conserved regions I–V. These correspond to codons 13–19, 120–143, 172–182, 238–259 and 271–290, respectively; 86% of mutations are located between codons 120 and 290, and a high frequency of mutations are found at the "hot-spots", codons 175, 248 and 273 (LEVINE et al. 1994).

At present, more than 2500 mutations of the *p53* gene in human cancers or cell lines have been reported (GREENBLATT et al. 1994).

Most *p53* gene alterations are missense mutations. The mutant p53 proteins mostly have a much longer half-life than the wild-type protein, which permits detection by immunohistochemistry with the aid of the appropriate antibodies (Fig. 1). This finding has led to an immense amount of data on the occurrence of the p53 protein in human cancer (SOUSSI et al. 1994). However, uncritically equating presence of immunohistochemically detectable p53 protein with a mutant genotype is unwarranted, as it seems that stabilisation of the p53 protein may occur without *p53* gene mutations (WYNFORD-THOMAS 1992; BATTIFORA 1994; HALL and LANE 1994).

The discrepancies between the presence of *p53* mutations and negative p53 protein staining may be due to a mutation leading to a premature stop codon or to mutations without amino acid substitution. The presence of immunohistochemically detectable p53 protein without detectable *p53* gene mutations has been explained as follows. Firstly, it is possible that mutations are located outside of the most commonly analyzed part of the *p53* gene, exons 5–8. Secondly, the p53 protein may accumulate at levels sufficient to be detected by immunohistochemistry due to stabilisation induced by viral or cellular proteins. Thirdly, defects in other p53-responsive cell cycle regulators, such as the cyclin-dependent kinase inhibitor gene *p21* WAF-1/CIP-1, may lead to cell division not inactivated by p53 protein produced at a normal level, which the cell attempts to compensate by overexpression (MELHEM et al. 1995).

Obtaining real insight into the status of the *p53* gene and its protein in an individual lesion requires molecular biological analysis of the gene, which includes amplifying the gene by polymerase chain reaction (PCR) followed by the use of

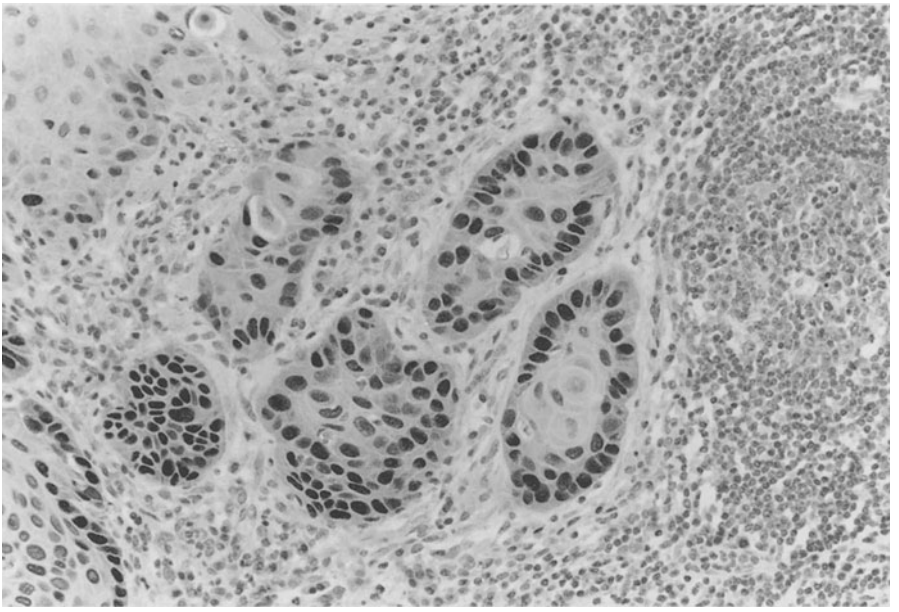

**Fig. 1.** Photomicrograph shows strong positivity for p53 protein in the nuclei of this squamous cell carcinoma. Haematoxylin–anti-p53 protein peroxidase–anti-peroxidase stain

techniques aimed at detecting altered electrophoretic mobility patterns, such as single-strand conformation polymorphism analysis (SSCP) or denaturant gradient gel electrophoresis (DGGE), followed by sequencing to detect the site of the mutation and detection of the protein by immunohistochemistry (Greenblatt et al. 1994; Soussi et al. 1994). Analysis for loss of heterozygosity at the *p53* locus completes the picture (Adamson et al. 1994).

## 5 p53 and Oral Tumours

### 5.1 Expression of p53 and *p53* Mutations

Data on *p53* gene alterations in oral tumours mainly concern squamous cell carcinomas (SCC) and their cell lines (Sakai et al. 1992; Sakai and Tsuchida 1992). In these tumours, *p53* gene mutations and the presence of p53 protein are consistent findings, although the proportion of SCC exhibiting these features varies in different series (Field et al. 1993a). The relationship between expression of detectable p53 protein and gene mutations has been investigated by combined immunohistochemical and molecular biological analysis. Some of these studies provided support for the assumption that expression of a stabilised and therefore detectable p53 protein is indeed correlated with a gene mutation (Gusterson et al. 1991;

Somers et al. 1992; Burns et al. 1993; Caamano et al. 1993; Ahomadegbe et al. 1995). In other studies, however, discrepancies between protein immunohistochemistry and gene analysis became apparent, indicating overexpression of p53 protein without apparent gene mutations (Ranasinghe et al. 1993a; Xu et al. 1994a,b; Melhem et al. 1995) as well as occurrence of gene mutations without p53 protein overexpression (Chen et al. 1994; Melhem et al. 1995).

Thus overexpression of p53 protein in oral SCC is not necessarily synonymous with *p53* gene mutations. Nevertheless, in general there is a correlation between detectable expression of p53 protein and neoplasia (Hall and Lane 1994), and therefore both data concerning p53 protein overexpression and studies in which gene alterations have been investigated will be reviewed.

## 5.2 Association of p53 Status with Risk Factors for Oral Cancer

Elevated p53 protein expression has been attributed to gene alterations induced by smoking, a well-known risk factor for oral SCC in Western countries (Field et al. 1991, 1994; Langdon and Partridge 1992; Ogden et al. 1992; Gallo et al. 1995a; Gallo and Bianchi 1995; Tsuji et al. 1995). These findings agree very well with similar data obtained for SCC of the lung, another tobacco-induced cancer, in which *p53* gene alterations play a prominent role (Greenblatt et al. 1994). In contrast, no association between p53 protein overexpression and tobacco smoking was reported by Matthews et al. (1993), Högmo et al. (1994), Franceschi et al. (1995) and Nakanishi et al. (1995). Differences in studied populations or anti-p53 antibodies used cannot explain these contradictory results, as similar divergent data come from molecular biological studies. By sequencing the *p53* gene, it was demonstrated that smoking increased the frequency of *p53* gene mutations found in head and neck SCC (Brennan et al. 1995a; Koch et al. 1995) and in pre-malignant lesions (Lazarus et al. 1995), whereas it was also found that the spectrum of types of *p53* gene mutations in non-smokers was limited to sites characteristically seen with spontaneous mutations, the hotspots, while those seen in smokers were more widely distributed (Brennan et al. 1995a; Koch et al. 1995). On the other hand, Yeudall et al. (1995), who performed studies on SCC cell lines, failed to observe this correlation between the nature of *p53* gene mutations and the use of tobacco by the patients from which the cell lines were derived.

Nevertheless, after combining the high proportion of *p53* gene mutations in head and neck SCC with the epidemiological evidence that smoking is an aetiological factor in this kind of tumour, one is led to the conclusion that the induction of *p53* gene alterations might be a way by which tobacco exerts its deleterious influence on the oral mucosa.

Whether *p53* gene mutations may be caused by betel quid chewing, a habit considered to play a prominent role in causing oral SCC in Asian populations, is also a subject of debate. Investigations in patients from Sri Lanka (Ranasinghe et al. 1993a,b) and from Papua New Guinea (Thomas et al. 1994) showed a low prevalence of *p53* gene alterations and p53 protein overexpression in betel-

associated oral cancers. However, in a study on patients from Northern India, the opposite was found (KAUR et al. 1994). Different chewing habits and differences in the ingredients of the quid in the various populations are probably responsible for the divergent data on the aetiological significance of betel quid chewing and *p53* gene alterations in the development of oral SCC (KUTTAN et al. 1995).

Abrogation of p53 function may also be caused by HPV, another risk factor associated with the development of oral SCC (YEUDALL 1992), as it has been shown that HPV-16 and HPV-18 E6 proteins form a complex and promote the degradation of cellular p53 protein (SCHEFFNER et al. 1990; WERNESS et al. 1990; LI et al. 1992). These observations prompted several authors to study the association between p53 status and HPV in oral SCC. BRACHMAN et al. (1992) were the first to investigative head and neck SCC for both *p53* gene mutations and the presence of HPV DNA and, as they did not find *p53* gene mutations and HPV DNA in the same tumour, they concluded that alteration of *p53* gene function in oral SCC may occur through gene mutations as well as HPV infection. MIN et al. (1994) found an inverse relationship between *p53* gene mutations and the presence of HPV in oral cancer cell lines; moreover, these authors reported a lower level of p53 protein than normal in cell lines expressing HPV-18 E6/E7 genes. Finally, an inverse relationship between p53 protein overexpression and HPV antigen detection in oral SCC has been reported by MUKHOPADHYAY et al. (1994).

Although these data do indeed suggest the involvement of HPV-triggered p53 protein degradation in oral carcinogenesis, matters may be more complicated, as SNIJDERS et al. (1994) and LEWENSOHN-FUCHS et al. (1994) found evidence for p53 protein stabilisation rather than increased degradation in some cases of tonsillar SCC containing HPV. Therefore, it might well be that HPV can interfere with normal p53 functioning by protein degradation as well as by binding to an HPV-derived viral protein. Evidence of HPV-mediated p53 protein stabilisation has also been provided by DEMERS et al. (1994). Finally, YEUDALL et al. (1995) demonstrated that SCC cell lines may express mutant p53 protein and also harbour HPV DNA, and they speculate that cells transformed by HPV may become further transformed by co-expression of mutant p53.

In summary, HPV may be a risk factor associated with oral SCC by disturbing p53 function, but its mode of operation is still far from clear.

## 5.3 Alterations of *p53* in Carcinogenesis

### 5.3.1 Timing

There is ample evidence that oral SCC may be preceded by pre-malignant mucosal changes, but the molecular events associated with the development of malignancy are poorly understood. Determination of the timing of p53 changes during the multistage process of progression from normal epithelium through dysplasia to invasive carcinoma may be helpful in understanding the genetic alterations which give rise to pre-malignant oral mucosal lesions and which influence their progression.

Timing of *p53* changes in the development of SCC has mainly been investigated by immunohistochemical analysis of p53 protein overexpression in oral mucosal dysplasia found adjacent to invasive carcinoma or in dysplastic lesions without an invasive component. GUSTERSON and colleagues (1991) were the first to report expression of p53 protein in oral SCC as well as in areas of adjacent mucosal dysplasia, with cytologically normal epithelial cells being negative (Figs. 2, 3). These findings have since been confirmed several times (WARNAKULASURIYA and JOHNSON 1992; NEES et al. 1993; PAVELIC et al. 1992, 1994; SLOOTWEG et al. 1994; XU et al. 1994a; GALLO et al. 1995a; LAVIEILLE et al. 1995). Moreover, expression of p53 protein has been observed in dysplastic lesions without a concomitant invasive component (COLTRERA et al. 1992; LANGDON and PARTRIDGE 1992; WARNAKULASURIYA and JOHNSON 1992; NAKANISHI et al. 1993; NISHIOKA et al. 1993; KAUR et al. 1994; PAVELIC et al. 1994) and in dysplastic lesions that subsequently developed into SCC (REGEZI et al. 1995). SAUTER et al. (1994) and SHIN et al. (1994) contributed to these data by reporting that the frequency of p53 protein expression was increased in lesions showing moderate or severe dysplasia compared to mildly dysplastic lesions. Occurrence of p53 overexpression in normal epithelium adjacent to tumour was reported by NEES et al. (1993), SAUTER et al. (1994), SHIN et al. (1994), AHOMADEGBE et al. (1995), GALLO and Bianchi (1995) and NAKANISHI et al. (1995).

Occurrence of p53 protein overexpression in normal control epithelium taken from cancer-free individuals was reported by GIROD et al. (1993, 1994a,b), who observed p53 protein positively in patients with lichen planus. However, lichen

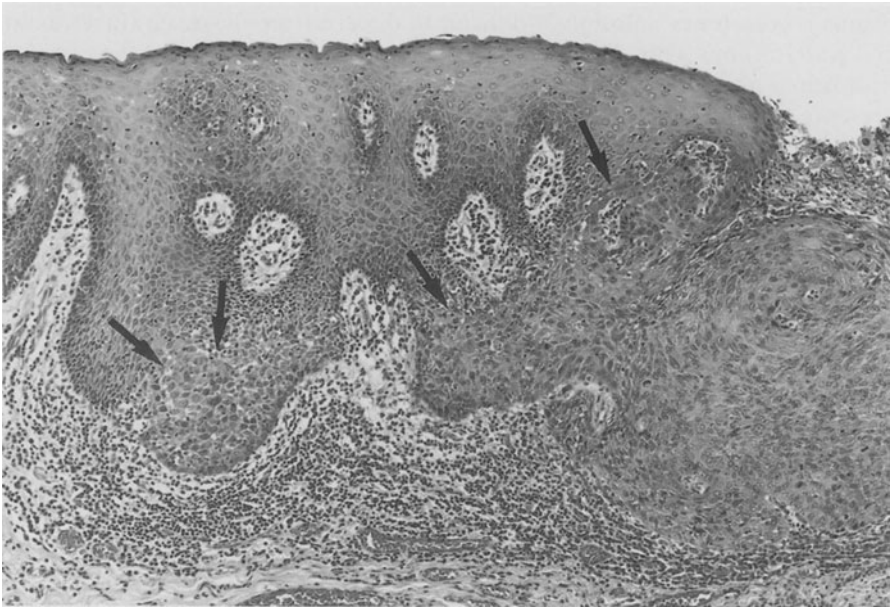

**Fig. 2.** Photomicrograph shows border between dysplastic and healthy oral epithelium; border indicated by *arrows*. H&E

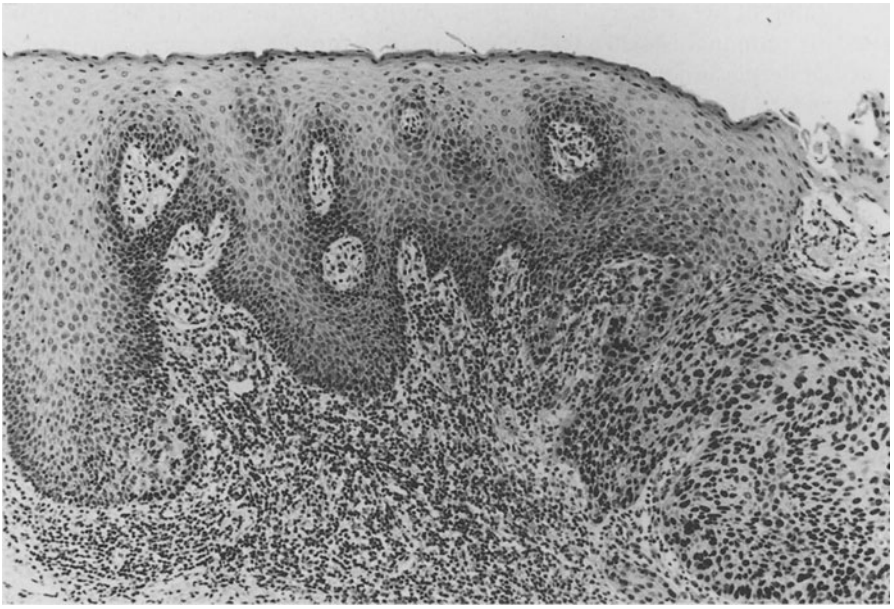

**Fig. 3.** Same area as shown in Fig. 2. Cells in the dysplastic epithelium exhibit positivity for p53 protein, which indicates protein overexpression. Haematoxylin–anti-p53 protein peroxidase–anti-peroxidase stain

planus is sometimes notoriously difficult to discern from dysplasia (Eisenberg and Krutchkoff 1992; Holmstrup 1992), and the possibility that the patients with lichen planus in reality suffered from lichenoid dysplasia may explain the presence of p53-positive cells in these cases. Overexpresion of p53 protein in cancer-free control tissue was also observed by Gallo and Bianchi(1995), who observed a few positive cells in patients with chronic inflammation. To clarify this issue, more studies on the association between p53 protein positivity and inflammatory mucosal disorders are needed.

All these data from which a derangement of p53 function early in cancer development can be inferred come from studies in which overexpression of p53 protein has been employed as an indication for *p53* gene mutations which are not necessarily synonymous with each other, as illustrated by the observation that a positive immunoreaction in dysplasia might reflect nuclear accumulation of wild-type p53 protein (Nakanishi et al. 1995). However, evidence that *p53* gene mutations, and not merely wild-type p53 protein overexpression, are associated with the development of dysplasia comes from several molecular biological studies. Boyle et al. (1993) and Lazarus et al. (1995) observed *p53* gene mutations in non-invasive lesions, from which they inferred that *p53* mutations could precede invasion in oral SCC. These observations were extended by Nees et al. (1993), who found *p53* gene mutations in histologically inconspicuous epithelia at a significant distance from the primary tumour, thus demonstrating that an occasional p53 protein-positive cell in an otherwise normal epithelium from a patient with oral

SCC may indeed harbour a mutated *p53* gene. Both the observations of BOYLE et al. (1993) and LAZARUS et al. (1995) that *p53* mutations occur in pre-invasive lesions and the finding of NEES et al. (1993) that tumour-distant epithelial areas may harbour *p53* mutations different from the invasive tumour argue against the idea that the presence of p53 protein-positive cells in tumour-adjacent mucosa may be due to intra-epithelial lateral spread of tumour cells and demonstrated that p53 protein positivity in non-invasive lesions may indeed denote the presence of gene mutations.

Moreover, evidence that *p53* gene mutations already occur during the transition from normal epithelium to dysplastic epithelium comes from a study by BURNS et al. (1994a) on the p53 status of cultured human pre-malignant keratinocytes in which they demonstrated elevated levels of p53 protein together with gene mutations.

Two studies suggest that *p53* gene changes are a late event in oral carcinogenesis. The first of these was carried out by CAAMANO et al. (1993), who base their opinion on the observation that the clinically furthest advanced tumours exhibit the most intense p53 protein immunostaining and that tumours showing the most invasive growth in an vivo assay employing growth of tumour explants in rat tracheal grafts also exhibit the most intense p53 protein expression. The other study providing support for *p53* alterations as a late event comes from LEE et al. (1993), who were only able to demonstrate *p53* gene mutations in invasive tumours, but not in adjacent tissue. However, no dysplasias were investigated.

In spite of these two reports, data from other studies mentioned above provide overwhelming evidence that *p53* alterations are an early event in oral carcinogenesis, involved in the development of pre-malignant mucosal changes.

Studies on the association between p53 protein overexpression in oral pre-malignant lesions and other histologically detectable features indicative of disturbed proliferation and differentiation are scarce. GIROD et al. (1994a,b) reported that proliferating cell nuclear antigen (PCNA) expression in pre-malignant lesions of the oral mucosa correlated with the dysplasia grade but did not quantify PCNA expression in correlation with p53 protein expression. NEES et al. (1993) observed p53 protein expression in tumour-adjacent epithelium to be associated with increased proliferation, as measured by analysis of expression of the histone H3 gene. However, whether this phenomenon also occurred in p53 protein-negative specimens of tumour-adjacent epithelium was not mentioned. As SLOOTWEG et al. (1994) found no quantitative differences in proliferation, as measured by Ki-67 reactivity between p53 protein-positive and p53 protein-negative tumour-adjacent dysplastic epithelia, it can be argued that p53 protein expression is related to dysplasia-associated increased cellular proliferation and development of cytological atypia but is not a condition sine qua non, as histologically similar epithelial dysplastic changes can be found without p53 protein expression.

To obtain more insight in the role of *p53* alterations in the development of mucosal pre-malignancies, studies are needed in which p53 protein expression is compared with changes in proliferation rates and DNA ploidy, as was done for bronchial mucosa, in which DNA aneuploidy was preceded by increased proliferation and followed by p53 protein immunoreactivity (HIRANO et al. 1994).

Moreover, the recently described association between *p53* gene changes and angiogenesis needs to be explored. As reviewed by WEIDNER (1995), angiogenesis plays a key role in tumour growth, progression and metastasis, and there are some indications that a normal *p53* gene inhibits angiogenesis (GASPARINI 1995). As angiogenesis is more related to advanced tumour growth than to early cellular events, an involvement of the *p53* gene with this phenomenon would mean that *p53* disturbances play a role not only in tumour initiation, but also in tumour progression and development of metastatic deposits. Up to now, only one report has paid attention to the relationship between p53 protein overexpression and intratumoral microvessel density; in this study, a significant association between the two features was observed (GASPARINI et al. 1993). One other study has also been devoted to p53 protein overexpression and tumour angiogenesis (LEEDY et al. 1994), but without investigating their mutual association. Whether *p53* gene changes are important in more advanced SCC will be an important item for future investigations; at present, their role in early stages of carcinogenesis has been better analyzed.

### 5.3.2 Use as a Biomarker

As concluded in the previous section, *p53* gene alterations occur early in oral carcinogenesis and may be visualised by immunohistochemical demonstration of p53 protein. Whether p53 protein serves as a useful biomarker will now be discussed.

A useful biomarker should fulfil two requirements. Firstly, the purported biomarker should be present in cells which do not yet show conventional histological signs of malignancy, such as cytonuclear atypia, but nevertheless are already at risk for malignant degeneration, as shown by subsequent development of dysplasia or carcinoma. As most studies indicate p53 protein overexpression in cells already exhibiting atypia or in normal cells from patients already suffering from SCC (references as cited in the previous section), p53 protein does not appear to be a useful biomarker in predicting the occurrence of oral SCC in patients not yet showing dysplasia or SCC. Matters may be different with patients already suffering from oral SCC, as there are some indications that in this group p53 protein overexpression in tumour-adjacent normal epithelium may indicate an increased risk for the development of multiple primary SCC of the upper aerodigestive tract (GALLO and BIANCHI 1995; NAKANISHI et al. 1995), carcinogens possibly interacting with a genetically determined increased mutagen sensitivity in these patients (GALLO et al. 1995a).

Secondly, a biomarker should be useful in identifying dysplasias at risk of progressing to invasive SCC. For p53 protein, this would imply that p53 protein-positive dysplastic lesions should have an increased risk of further malignant degeneration in comparison with p53 protein-negative cases of dysplasia. This has not so far been found; in contrast, REGEZI et al. (1995) observed that both p53 protein-positive and p53 protein-negative dysplasias developed into SCC. Moreover, *p53* gene alterations occur early in oral carcinogenesis, but are not associated with disease progression (AHOMADEGBE et al. 1995). Therefore, it

would be illogical to suppose that p53 protein was a biomarker able to identify dysplastic lesions with an increased risk of developing into SCC, as this implies cellular events connected with the later stages of oral carcinogenesis in which *p53* gene alterations are probably not involved. In summary, p53 protein may be useful in identifying SCC patients at risk of developing a second malignant primary tumour, but it appears not to be of any use in identifying tumour-free patients at risk of developing dysplasia or SCC or patients with dysplasia at risk of progressing to SCC.

## 5.4 Clinical Utility of p53 Analysis

### 5.4.1 Diagnostic Applications

Mutations in the *p53* gene may occur at different sites, a feature which may have diagnostic value in discerning between multiple primary tumours and recurrent or metastatic disease.

In the event of multiple primary tumours, different *p53* gene mutations can be found in the respective lesions, as has repeatedly been demonstrated (CHUNG et al. 1993; KOCH et al. 1994; ZARILAWA et al. 1994), whereas metastatic or recurrent tumours should retain the same mutation. As almost all head and neck cancers are of the squamous cell type, analysis of histological features is of no use in solving this diagnostic problem, and therefore availability of a non-morphological tool to identify tumours would be very useful, as treatment modalities for patients with a second primary oral SCC may be entirely different from those for patients with recurrent or metastatic disease.

The idea that *p53* gene mutations are maintained during tumour progression and metastasis has gained support from studies on primary SCC and their lymph node metastases. Results from these investigations can be summarised as follows. CHUNG et al. (1993) analysed three cases of primary SCC of the head, neck and lung and their respective regional lymph node metastases and noted identical mutations in primary tumour and metastasis exhibited by identical SSCP banding; no sequencing data were given. BURNS et al. (1994b) reported data using the technique of sequencing which demonstrated conservation of the *p53* gene mutation throughout progression to lymph node metastasis. KOCH et al. (1994) reported different mutations in two synchronous primary oral SCC; bilateral lymph node metastases contained the same mutated sequence as found in one of the primary SCC. AHOMADEGBE et al. (1995) observed identical mutations in primary tumour and lymph node metastasis in eight out of ten patients, whereas a mutation was only observed in the node metastasis in two patients. These studies suggest that mutant *p53* genes may indeed be useful in distinguishing between SCC and metastasis and/or recurrent disease.

Less clear-cut results were obtained by ZARILAWA et al. (1994), who reported a patient with a *p53* gene mutation in a lymph node metastasis not detected in the primary malignancy, a finding they explained by assuming that the mutation in the lymph node metastasis arose independently after metastatic spread or that muta-

tion was present in the primary tumour in such a small fraction that it escaped detection.

As different *p53* gene mutations have been observed even within single biopsies of head and neck SCC (NEES et al. 1993), the presence of tumour clones in the primary lesion too small to be detected but with a higher propensity for metastasis may indeed explain differences in *p53* gene mutations between primary SCC and metastatic tumour deposits; therefore, the preliminary conclusion has to be that the same *p53* gene mutation present in tumours from several sites suggests one primary tumour that has metastasised, but that the presence of different mutations does not unequivocally rule out metastasis.

The value of *p53* gene mutation analysis as a reliable diagnostic tool to distinguish between multiple primary tumours and metastasising disease depends on the possibility of assessing whether an individual tumour harbours a single *p53* gene mutation or several clones with different *p53* gene mutations, even if some of these are only represented by a few tumour cells.

BRENNAN et al. (1995b) showed that application of such techniques yields clinically useful data; they demonstrated the presence of tumour cells not otherwise detected in surgical margins and excised lymph nodes from SCC patients by employing p53 mutant-specific probes.

### 5.4.2 Prognostic Significance

p53 protein is involved in control of genomic stability and cell cycle control in response to DNA damage. Loss of *p53* function (often seen as high p53 protein levels) would therefore be expected to correlate with aneuploidy and increased proliferation rates (GREENBLATT et al. 1994; HARTWELL and KASTAN 1994; BATSAKIS and EL-NAGGAR 1995). As these two features may be associated with poor prognosis, determination of p53 protein overexpression, although an indirect and admittedly imperfect means of estimating *p53* gene mutations, could be helpful in predicting tumour behaviour.

This assumption has been tested in various ways. LOWE et al. (1994) compared the therapeutic responsiveness of genetically defined tumours expression or devoid of the *p53* gene in mice. They found that tumours expressing the *p53* gene regressed after irradiation or chemotherapy, whereas *p53*-deficient tumours continued to enlarge; they therefore concluded that defects in *p53* function may be a significant impediment to successful cancer therapy. This study agrees very well with those of LIU et al. (1994, 1995) and CLAYMAN et al. (1995), who demonstrated that introduction of wild-type p53 into SCC cell lines via a recombinant adenoviral vector resulted in growth arrest and morphological changes consistent with apoptosis in vitro as well as significant tumour reduction in in vivo studies on nude mice. The influence of *p53* gene mutations on response to irradiation was analysed in SCC cell lines. JUNG et al. (1992) investigated whether *p53* mutations correlated with either a radiation-sensitive or radiation-resistant cellular phenotype, but did not find any difference. BRACHMAN et al. (1993), however, observed a trend toward increased radioresistance in SCC cell lines with abrogation of *p53* function.

It appears from these basic studies that normal *p53* functioning might have a beneficial effect on cancer treatment, probably by inducing apoptosis in the presence of oncogenic triggers (FISHER 1994). However, proof has to come from patient studies, which will be discussed in the following.

The value of p53 protein overexpression, as determined by immunohistochemistry, as a prognostic factor has been investigated by analysing correlations between this feature and clinico-pathological tumour parameters such as size, stage and histological grade by comparing p53 protein overexpression with cell-kinetic data and by evaluating the relationship between p53 protein overexpression and survival time.

The reports that correlate p53 protein immunohistochemistry with tumour cell kinetics will first be mentioned. BOURHIS et al. (1994) employed DNA flow cytometry after in vivo bromodeoxyuridine labelling and p53 protein immunohistochemistry. Their data suggest that overexpression of p53 protein is significantly more frequent in aneuploid tumours and significantly associated with a high proportion of cells in S phase. The authors conclude that overexpression of the *p53* gene is associated with rapid tumour cell proliferation. In contrast, HÖGMO et al. (1994) and TSUJI et al. (1995) failed to find a correlation between p53 immunostaining and DNA aberration, and MUKHOPADHYAY et al. (1994), who related p53 protein overexpression to in vitro bromodeoxyuridine labelling, observed no correlation between p53 positivity and cytokinetics in their series. WILSON et al. (1995) found no differences in DNA aneuploidy and proliferation rate measured by in vivo bromodeoxyuridine between p53 protein-positive and p53 protein-negative cases; however, when differentiating between strongly and weakly positive cases, the strongly positive tumours were more often aneuploid. NYLANDER et al. (1995a,b) found no correlation between *p53* gene mutations and p53 protein overexpression on the one hand and in vivo incorporation of iododeoxyuridine or number of cells expressing the immunohistochemically detectable proliferation marker PCNA on the other. A similar lack of correlation between p53 protein overexpression and expression of PCNA was observed by LAVIEILLE et al. (1995) and earlier by WARNAKULASURIYA and JOHNSON (1994), but with another proliferation marker, Ki-67, the latter authors observed an increased proportion of Ki-67-labelled cells in p53 protein-positive carcinomas. A positive correlation between p53 protein overexpression and the number of PCNA-positive cells was reported by NISHIOKA et al. (1993) and TSUJI et al. (1995).

Summarising the as yet rather limited data on the relationship between p53 protein overexpression in oral SCC, their proliferation rate and their ploidy status, it appears that this issue is far from clear. The cell-kinetic data obtained so far in various ways do not provide unequivocal support for the idea that p53 protein overexpression is associated with an increased proliferation rate or aneuploidy.

Another way to investigate whether p53 protein overexpression has prognostic significance is by analysing the association of this feature with other clinicopathologic parameters influencing clinical outcome, such as histological grade, tumour size, clinical stage and presence of metastatic disease. Although some authors observed an association between p53 protein overexpression and tumour

size as well as metastatic disease (GASPARINI et al. 1993) or between this feature and metastatic disease only (BOURHIS et al. 1994), most other authors do not confirm these findings, as no correlation between p53 protein overexpression and tumour stage, including metastatic disease, was found in their investigations (FIELD et al. 1991, 1993b, 1994; PAVELIC et al. 1992; GAPANY et al. 1993; NISHIOKA et al. 1993; HÖGMO et al. 1994; LEEDY et al. 1994; SCHIPPER and KELKER 1994; XU et al. 1994; AHOMADEGBE et al. 1995; BRENNAN et al. 1995a; LAVIEILLE et al. 1995; NAKANISHI et al. 1995; NYLANDER et al. 1995a,b). An inverse relationship between p53 protein overexpression and stage has even been reported (MUKHOPADHYAY et al. 1994).

As far as association of p53 protein overexpression and histological tumour features are concerned, data are as divergent as for clinical tumour parameters. No correlation between the presence of p53 protein and histological grade was observed by FIELD et al. (1991), PAVELIC et al. (1992), GAPANY et al. (1993), HÖGMO et al. (1994), SCHIPPER and KELKER (1994), XU et al. (1994a) BRENNAN et al. (1995a) or NAKANISHI et al. (1995). MUKHOPADHYAY et al. (1994) observed an inverse relationship between p53 protein overexpression and histological grade, p53 protein being most predominant in well-differentiated tumours. WATLING et al. (1992), however, found overexpression of p53 protein to be strongly associated with a histological malignancy grading scale, and LEE et al. (1993) found that p53 protein expression correlated with deeply invasive tumour growth.

Another way by which *p53* gene mutations might influence prognosis has been proposed by HALD et al. (1994). These authors argued that mutated p53 proteins could be targets for cytotoxic T lymphocytes, thus inducing a possibly beneficial immune response of host to tumour. However, as peri-tumoural T cell infiltration did not correlate to p53 protein overexpression, T cell infiltration probably does not reflect an activation process induced by mutated p53 proteins, and it may be conluded that these mutated p53 proteins do not confer the immunogenic properties to tumour cells that are required to elicit a T cell-mediated cytotoxic attack.

The most conclusive evidence that *p53* gene alterations are associated with prognosis needs to be provided by studies on patient survival. Such studies are few. FIELD et al. (1993b, 1994) noted no differences in survival time between patients with p53 protein-positive SCC and those with p53 protein-negative SCC, while AHOMADEGBE et al. (1995) failed to observe a different survival time between SCC patients with *p53* gene mutations and those without. When comparing the outcome of radiotherapy in SCC patients with and without p53 protein overexpression, no differences were observed (WILSON et al. 1995).

BRACHMAN et al. (1992) observed that those patients with tumours in which *p53* DNA sequence changes were detected had a shorter time to recurrence at the primary tumour site than those patients whose tumours had no *p53* gene mutations. This trend towards earlier disease recurrence did not appear to be associated with tumour stage or type of treatment. A similar adverse influence of *p53* gene alterations on survival was observed by LEE et al. (1993), who reported an increased risk of death from disease in patients with *p53* gene mutations compared with patients without *p53* gene mutations, and by GLUCKMAN et al. (1994) and TSUJI et al. (1995), who reported an association between p53 protein overexpression and decreased survival.

In contrast with these reports, SAUTER et al. (1992) observed that patients with p53 protein overexpression had a longer mean survival time than those without. They concluded that p53 protein overexpression may serve as a marker indicative of improved survival potential, whereas HÖGMO et al. (1994) failed to establish a significant impact of p53 positivity on prediction of survival.

Finally, it should be mentioned that there appears to be an association between p53 protein overexpression and response to chemotherapy – either negative, as observed by FIELD et al. (1993b), who reported an association between p53 protein overexpression and poor clinical outcome in a group of SCC patients with end-stage disease, or positive, as found by LAVIEILLE et al. (1995), who reported a significant correlation between complete response to induction chemotherapy and a high percentage of p53 staining in biopsies obtained before treatment.

From all these studies devoted to analysis of the influence of *p53* gene alterations on prognosis concerning oral SCC, irrespective of whether one considers cell kinetics, clinico-pathological parameters or disease-free survival time, no clear-cut picture emerges to indicate either a positive or a negative influence. It appears that so far detection of *p53* gene alterations has not fulfilled the characteristics of a useful prognostic factor as summarized by BATSAKIS and EL NAGGAR (1995): (a) significant and independent predictive value validated by clinical testing; (b) able to be determined by methods that are feasible, reproducible and widely available with appropriate quality control; (c) readily interpretable with clinical and therapeutic implications.

## 5.5 Alterations of *p53* in Oral Tumours Other Than Squamous Cell Carcinoma

Most data on *p53* gene alterations and p53 protein overexpression in oral tumours come from studies on SCC; much less attention has been paid to other oral tumours. In fact, the only studies performed have been devoted to p53 and salivary gland tumours; most of these concern pleomorphic adenomas and their malignant counterparts.

Overexpression of p53 protein was detected in benign pleomorphic adenomas and cell lines derived from this tumour (AZUMA et al. 1992; SOINI et al. 1992; DEGUCHI et al. 1993; RIGHI et al. 1994; GALLO et al. 1995b).

Some authors compared the expression of p53 protein in benign pleomorphic adenomas and carcinomas arising from pleomorphic adenoma. In general, the malignant tumours exhibited stronger positivity as well as more positive cells than the benign ones (GALLO et al. 1995b), and it has been proposed that involvement of p53 mutation may play an important role in the malignant transformation of salivary gland pleomorphic adenoma (DEGUCHI et al. 1993; RIGHI et al. 1994). As far as other salivary gland tumours are concerned, p53 protein overexpression has been reported to occur in muco-epidermoid carcinomas and undifferentiated carcinomas (SOINI et al. 1992), salivary duct carcinomas (HELLQUIST et al. 1994; ISHII and NAKAJIMA 1994; LI et al. 1995), adenoid cystic carcinomas, acinic cell

carcinomas, polymorphous low-grade adenocarcinomas and epithelial myoepithelial carcinomas (GALLO et al. 1995b).

Few data are available on correlations of p53 protein overexpression with other clinico-pathological features. SOINI et al. (1992) reported that in malignant salivary gland tumours the p53 protein positivity tended to be more evident in less-differentiated tumours. ISHII and NAKAJIMA (1994) reported a close correlation between p53 protein positivity and DNA aneuploidy in high-grade carcinomas of salivary gland tissue and, according to GALLO et al. (1995b), p53 protein positivity is an independent indication of clinical aggressiveness in patients with carcinoma of the parotid gland, as indicated by its correlation with regional and distant metastatic disease and a lower survival rate. It appears that p53 protein overexpression occurs in a variety of malignant salivary gland tumours. Its purported predictive role as a marker of incipient malignancy in pleomorphic adenoma and its prognostic significance in patients with salivary gland carcinomas both warrant further investigations.

## 5.6 Concluding Remarks

A vast amount of data on *p53* gene function, mutation and protein overexpression and its association with clinico-pathological parameters has appeared in the literature during the past few years. As will have become apparent from the foregoing, *p53* gene alterations in oral SCC are frequently found and occur early in malignant transformation; it may be expected that further studies will augment our knowledge about the way in which *p53* is connected with regulation of cellular proliferation and differentiation.

Whether determining *p53* gene status will provide useful knowledge applicable to clinical practice in the near future is debatable.

In a diagnostic sense, *p53*-mutant specific probes may represent a tool to detect tumour cells that are not visualised by conventional histological techniques, as outlined by BRENNAN et al. (1995b). Moreover, analysis of *p53* gene mutations by sequencing may be helpful in distinguishing multiple primary tumours from recurrent or metastatic disease, although more extensive application of this technique requires evaluation of the prevalence of multiple *p53* gene mutations in one and the same lesion (NEES et al. 1993). Both DNA sequencing and preparation of mutant-specific probes, however, are not routinely employed in pathology, and they are very laborious. Therefore, it is not expected that they will be widely employed in the near future.

Overexpression of p53 protein as demonstrated by immunohistochemistry is more easily applicable in histopathological laboratories. However, its usefulness appears to be rather limited as far as diagnosis and prognosis are concerned, as has been discussed above. The confusing and contradictory data that have been published concerning impact of p53 protein overexpression on survival and the association of this feature with established clinico-pathological parameters is probably, at least in part, due to a lack of uniformity in immunohistochemical procedures.

Studies differ in the method of tissue preparation used (frozen versus paraffin-embedded material), in employing antibodies with different epitope specificities and in the use of antigen retrieval methods, all of which may not only influence the number of p53 protein-positive cases in a particular series, but also the number of p53 protein-labelled cells in an individual tumour (HALL and LANE 1994). Moreover, p53 protein positivity is scored in different ways. Methods vary from simply establishing whether there are any p53 protein-positive cells (GIROD et al. 1994a,b) to elaborate semi-quantitative methods in which both cell numbers and staining intensities are taken into account (PAVELIC et al. 1992).

This methodological diversity makes comparison of data from various series virtually impossible, and it will be apparent that it is necessary to formulate standard procedures for detection of p53 protein overexpression and positivity scoring in order to gain more insight into the significance of this feature in oral SCC.

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# Genomic Instability in Head and Neck Cancer

A.G.M. SCHOLES and J.K. FIELD



## 1 Introduction

Carcinogenesis is a multistage process, resulting from the accumulation of genetic alterations. Proliferation of normal cells is thought to be regulated by growth-promoting proto-oncogenes counterbalanced by growth-constraining tumour suppressor genes (TSG) (WEINBERG 1991). During tumour initiation and progression, proto-oncogenes may be activated by amplification, rearrangement or point mutation, whilst loss of function of TSG may be caused by deletion or mutation. Precisely how many genetic alterations are required for tumourigenesis is unclear; statistical analysis based on age-specific data suggests that five or six steps are generally necessary (RENAN 1993). In a molecular model specific for colorectal tumourigenesis, it has been proposed that mutations in at least four to five genes are required for the formation of a malignant tumour (FEARON and VOGELSTEIN 1990). In the case of head and neck cancer, however, it has been suggested that a greater number of genetic lesions are required (RENAN 1993), and it is evident from allelotype analysis that the process is complex (FIELD et al. 1995b).

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Determination of the critical genetic events in head and neck carcinogenesis will conceivably allow early detection and direct future treatment of these cancers. In this chapter, potentially important chromosome regions identified by cytogenetic and loss of heterozygosity (LOH) analysis in squamous cell carcinomas of the head and neck (SCCHN) will be reviewed. Recent evidence regarding the role of DNA repair genes in genomic instability and problems associated with the development of a molecular progression model for SCCHN will be discussed.

## 2 Cytogenetic Analysis in Squamous Cell Carcinoma of the head and Neck

Characterisation of particular chromosome aberrations in tumour cells by cytogenetic analysis has been the first step in identifying genes which may be involved in cancer development. These aberrations include whole chromosomal loss or gain, chromosomal rearrangements, such as inversions and translocations, deletions

**Table 1.** Summary of chromosome aberrations detected in head and neck squamous cell carcinomas by cytogenetic analysis

| Chromosome | Regions with aberrations |
|---|---|
| 1 | 1p, 1q, 1p11–p12, 1p13, 1p22, 1p36, 1q21, 1q25, 1q32 |
| 2 | 2q, 2q33–q36 |
| | 3p, 3q, 3pter–p23, 3p11, 3p13, 3p13–p23, 3p14–p25, 3p21, 3p21–qter, 3p26–qter, 3cent–qter |
| 4 | 4p, 4q, 4q11–q21, 4q21, 4q21–qter |
| 5 | 5p, 5p11, 5q, 5q12–q23 |
| 6 | 6q15–q26, 6q21–q25 |
| 7 | 7p, 7q, 7cent–p15, 7p11, 7p21–p22, 7q22–q34, 7q33–qter |
| 8 | 8p, 8q, 8p11.2, 8p22–p23, 8 cent, 8cent–q21.2, 8q10 |
| 9 | 9p, 9p21–p24, 9 cent, 9q32 |
| 10 | 10p, 10q, 10pter–q21.2, 10q11.2, 10q22–q26 |
| 11 | 11p, 11q, 11p15, 11q13, 11q13–q21, 11q13–q23, 11q23 |
| 12 | 12p, 12p11.2 |
| 13 | 13p, 13q, 13p11–p13, 13q32–qter |
| 14 | 14p, 14q, 14p11–q11 |
| 15 | 15p, 15p11–q11, 15q10 |
| 16 | 16p |
| 17 | 17p, 17q |
| 18 | 18q, 18q21, 18q21–qter, 18q22, 18q22–q23 |
| 19 | 19p, 19q, 19pter–cen, 19p13, 19p13.1 |
| 20 | 20p, 20p13 |
| 21 | 21p, 21q |
| 22 | 22p, 22q |

Compilation of aberrations detected by HAUSER-URFER and STAUFFER 1985; JIN et al. 1990; ALLEGRA et al. 1992; COWAN 1992; MITELMAN 1991; OSELLA et al. 1992; OWENS et al. 1992; JIN and MERTENS 1993; JIN et al. 1993; RAO et al. 1994; VAN DYKE et al. 1994; JIN et al. 1995 and SPEICHER et al. 1995.

and amplifications. Identification of chromosomal regions frequently deleted or duplicated may indicate possible sites of TSG or oncogenes, respectively; chromosomal bands frequently rearranged may represent sites of oncogene activation (Cowan 1992). Review of cytogenetic studies using cultures derived from SCCHN shows that the majority of autosomal chromosome arms may be affected (Table 1). A problem with the use of cell lines, however, is that the rearrangements observed may not be represented in the primary tumour. Indeed, a comparison of karyotypic changes in cell lines with those of the corresponding primary carcinoma identified clonal evolution in all cell lines (Sreekantaiah et al. 1994). This has been largely overcome by the use of short-term cultures, although different culture conditions may favour the proliferative growth of different cell populations (Jin et al. 1993). A consistent abnormality common to all SCCHN has not been identified, but it is clear that there is a recurrent clustering of aberrations on several chromosomal regions. In a study of short-term cultures from SCCHN, Jin et al. (1995) found the chromosomal bands most frequently involved were, in order of decreasing frequency, 8p11–q11, 1p11–q11, 3p11–q11, 11q13, 13p11–q11, 1p13, 5p11–q11, 7p11–q11, 15p11–q11 and 14p11–q11. It is notable that Cowan (1992) reported deletions of the following specific regions at high frequency: 3pter–p23, 4q11–q21, 4q11–q221, 4q21–qter, 8pter–p23, 8p23–p21 and 10pter-p11.2. In addition, chromosome region 7cent-p15 was duplicated at high frequency (Cowan 1992).

## 3 Allelic Imbalance

Analysis of allelic imbalance (i.e. LOH analysis) has a major advantage over cytogenetic techniques as it has a greatly enhanced resolution, especially if closely spaced microsatellite markers are used. This technique allows the determination of specific chromosome regions containing deletions (allelic loss), which has led to the identification of a number of TSG, including the retinoblastoma gene (RB) and adenomatous polyposis coli gene (APC) (Marshall 1991; Weinberg 1991).

Whilst early studies mainly used restriction fragment-length polymorphisms to detect allelic loss, recent studies have used microsatellite markers. Microsatellites are short, simple repeat sequences (typically di-, tri- or tetranucleotides) usually found in intergenic DNA or within the introns of genes. Dinucleotide repeats are very common, are relatively uniformly distributed throughout the genome and are often highly polymorphic as a result of variation in the number of repeated units from one allele to another. Polymerase chain reaction (PCR)-based amplification of specific microsatellite repeats can be carried out using primers which flank the area of interest (Weber and May 1989). Radioactively labelled products may be detected by autoradiography, following electrophoresis. Alternatively, the products may be visualised and sized on silver-stained acrylamide gels. Determination of allelic loss for informative markers is made by comparison of the relative intensity of normal and tumour alleles from the same patient. LOH may be determined by the absence of, or reduced, allelic amplification in the tumour

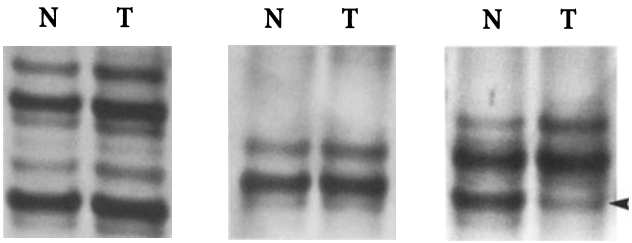


**Fig. 1a–c.** Representative allelic imbalance (loss of heterozygosity). **a** Retention of heterozygosity. **b** Homozygosity. **c** Allelic imbalance (*arrowhead*). "Stutter" or "shadow bands" may be seen in both the normal (*N*) and tumour (*T*) lanes. (From FIELD et al. 1995b, with permission)

sample. Complete loss is not always seen, due to contamination of the tumour with non-neoplastic cells (Fig. 1).

## 3.1 Possible Target Regions in Squamous Cell Carcinoma of the Head and Neck

Certain regions frequently show allelic imbalance in SCCHN, particularly on chromosome arms 3p, 5q, 9p, 9q, 11p, 11q, 13q, 17p and 18q (MAESTRO et al. 1993; ADAMSON et al. 1994; AH-SEE et al. 1994; LYDIATT et al. 1994; NAWROZ et al. 1994; SCHOLNICK et al. 1994; FIELD et al. 1995b; ROWLEY et al. 1995); these are detailed below. Other chromosomal regions which show LOH at lower frequency include 1p, 1q, 2p, 6p, 6q, 8p, 8q, 17q and 19q (FIELD et al. 1995b); although all of these are not discussed in detail, it is possible that genetic alterations at these sites may play an important role in the pathogenesis of certain tumours.

The findings reported in cytogenetic and allelic imbalance studies do not always agree; this may be due to the interpretation of LOH data. In general, papers reporting allelic imbalance define reduced intensity of one allele as indicative of LOH, although these studies cannot readily distinguish between allele deletion and low-level amplification or duplication. Thus apparent LOH may not necessarily be indicative of the presence of a TSG, and this should be taken into consideration when interpreting allelic imbalance studies (FIELD et al. 1995b; JIN et al. 1995).

### 3.1.1 Chromosome 1

Chromosome 1p frequently demonstrates cytogenetic abnormalities in SCCHN, particularly at 1p22 and 1p11–p12 (JIN et al. 1990, 1993; OWENS et al. 1992), whereas LOH analysis has demonstrated allelic loss of this chromosome arm in only 14%–30% of tumours (AH-SEE et al. 1994; NAWROZ et al. 1994; FIELD et al. 1995b). The study by FIELD et al. (1995b), however, used the greatest number of microsatellite markers and identified a minimal area of loss at 1p31.2–p21.3. Thus,

although loss of this region may not be as frequent as that at other chromosomal sites in SCCHN, retention of heterozygosity of markers outside this region in the majority of tumours suggests this chromosomal locus may be important in the pathogenesis of a number of head and neck tumours.

### 3.1.2 Chromosome 3

Allele loss on the short arm of chromosome 3 is common in SCCHN. Initial studies in early-passage cell lines defined the commonly deleted region as 3p14–26 (Latif et al. 1992). Later studies of paired tumour and normal mucosa have suggested the presence of at least three TSG on 3p. Maestro et al. (1993) demonstrated LOH at 3p in 74% of SCCHN and defined three regions of loss, 3p24–pter, 3p21.3 and 3p14–cen. In a study of oral carcinomas, Wu et al. (1994) identified LOH at 3p in 52% of tumours and mapped three distinct regions of loss, which appear to overlap with those described by Maestro et al. (1993), 3p13–p21.1, 3p21.3–p23 and 3p25. Other studies of SCCHN have found more than 60% loss using markers mapping within these regions, for example to 3p13–p14 and 3p21 (El-Naggar et al. 1993; Ah-See et al. 1994; Scholnick et al. 1994). Frequent deletion of these regions is not unique to SCCHN and has been demonstrated in many tumour types, including lung cancer (Hibi et al. 1992; Killary et al. 1992). A TSG locus at 3p21–p22, for example, has previously been defined, and a number of putative TSG have been isolated from this region (Killary et al. 1992). Recently, a human mismatch repair gene, *h*MLH1 (see Sect. 4) has been located on chromosome 3p21.3–p23 (Bronner et al. 1994). Studies of inactivation of these genes in SCCHN have not been reported.

The similar finding of three discrete regions of loss on chromosome 3p in lung cancer (Hibi et al. 1992) raises the possibility that aetiological factors common to both lung and head and neck cancer, such as smoking, may be associated with LOH on 3p. Our studies have found LOH on 3p to correlate with nodal metastases and TNM stage IV tumours (Field et al. 1994a), suggesting these alterations may occur as later events in the progression of SCCHN. Wu et al. (1994) showed that, in contrast to TNM stage I tumours, the majority of stage II–III oral carcinomas showed 3p LOH, although the sample size was small. Notable, however, was the finding that three out of the five stage IV tumours examined did not show loss, but two of these were from young subjects and were not associated with known aetiological factors; the third was associated with chewing of betel nut leaf quid (Wu et al. 1994).

### 3.1.3 Chromosome 5

The long arm of chromosome 5 contains the APC gene at 5q21, which has been linked with familial adenomatous polyposis (FAP) (Kinzler et al. 1991). FAP is characterised clinically by the presence of multiple polyps throughout the colon and rectum, at least one of which may become malignant. Allele loss on chromosome 5q has been demonstrated in 25%–43% of SCCHN, often involving the 5q21–q22 region (Ah-See et al. 1994; Field et al. 1995b). A study of oral SCC in Japan has

demonstrated LOH at the APC locus in 73%, but APC gene mutations in only 12.5% of tumours (UZAWA et al. 1994).

### 3.1.4 Chromosome 8

Cytogenetic studies of short-term cultures from head and neck carcinomas have reported frequent aberrations on chromosome 8, specifically at 8p11–q11, 8pter–p23 and 8p23–p21 (COWAN 1992; JIN et al. 1995). Frequent LOH (40%) has also been demonstrated on the p arm of this chromosome in SCCHN (KIARIS et al. 1994); an area of minimal loss was identified between markers D8S87 (8p12) and ANK1 (8p21.2–p11) (Fig. 2), suggesting the presence in this region of one or more putative TSG which may play a role in the development of these tumours. Chromosome 8p has not been examined in detail in SCCHN by other workers; only one microsatellite marker was used in the allelotype studies by AH-SEE et al. (1994) and NAWROZ et al. (1994). Studies of other tumour types, however, including colorectal, prostate, bladder, breast, lung and hepatocellular carcinomas, have also indicated the localisation of candidate TSG in the 8p22–p11 region (EMI et al. 1992; FUJIWARA et al. 1993; KNOWLES et al. 1993; TRAPMAN et al. 1994; KERANGUEVEN et al. 1995).

### 3.1.5 Chromosome 9

A common region of loss in SCCHN, seen in up to 72% of tumours and also in pre-invasive (severely dysplastic and carcinoma in situ) lesions, is at 9p21–p22 (LYDIATT et al. 1994; VAN DER RIET et al. 1994; EL-NAGGAR et al. 1995a). A high frequency of loss on chromosome 9p21–p23 has been demonstrated in a number of other tumour types, including non-small cell lung carcinoma (NSCLC) (MEAD et al. 1994; NOBORI et al. 1994; NEVILLE et al. 1995). The cyclin-dependent kinase-4 inhibitor genes, p15 and p16, which inhibit progression through the $G_1$ phase of the cell cycle, both map to 9p21. Deletions and mutations of these putative TSG have recently been reported in NSCLC (WASHIMI et al. 1995; XIAO et al. 1995). Sequence analysis of p16 in primary SCCHN with chromosome 9p loss has demonstrated that point mutations are infrequent (CAIRNS et al. 1994; ZHANG et al. 1994). The rarity of p16 point mutations does not necessarily indicate that p16 is not the target gene in tumours with 9p21 LOH. Using newly cloned markers around the p16 locus, CAIRNS et al. (1995) have convincingly demonstrated that small homozygous deletions represent an important mechanism of inactivation of 9p21 in many tumour types, including SCCHN. Fine mapping of these deletions implicates a 170-kb minimal region that includes p16 and excludes p15 (CAIRNS et al. 1995).

It has been suggested that an alternative mechanism for inactivation of p16 may be methylation of the 5′ CpG-rich region, which results in a complete block of gene transcription (MERLO et al. 1995). More than 70% LOH in SCCHN has also been reported at 9p22–q23.3, suggesting that this may be an additional TSG locus on chromosome 9p (NAWROZ et al. 1994).

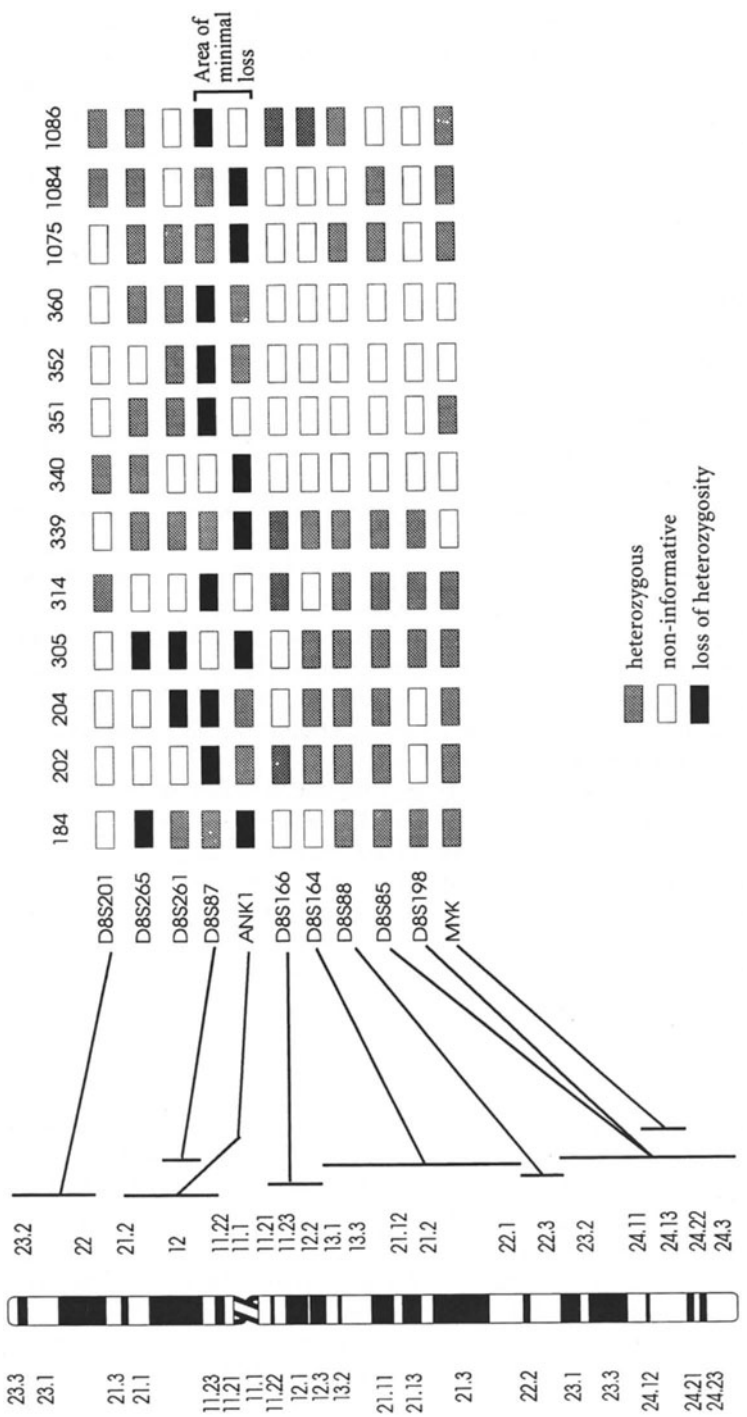


**Fig. 2.** Representative results of loss of heterozygosity on chromosome 8 in 13 patients exhibiting allele deletions with microsatellite markers D8S87 or ANK1. (From Kiaris et al. 1994, with permission)

Putative TSG located on the long arm of chromosome 9 include those related to Gorlin's syndrome (basal cell naevus syndrome) and the Ferguson-Smith syndrome (multiple self-healing squamous epitheliomata), mapped to 9q31 and 9q22–q31, respectively (GAILANI et al. 1992; GOUDIE et al. 1993). LOH on chromosome 9q has been found in 35% of SCCHN, at 9q31–q34 and 9q22.1–q32; thus it is possible that the gene or genes associated with these familial cancers may also be involved in some sporadic head and neck carcinomas (AH-SEE et al. 1994).

### 3.1.6 Chromosome 11

The short arm of chromosome 11 contains genes associated with Wilms' tumour, a childhood renal tumour with both a hereditary and a non-hereditary form. A candidate gene has been isolated at 11p13, although other genes in the region may play an important role in tumour development (GESSLER et al. 1990). LOH at 11p15.5 has been found in 50% of SCCHN, suggesting the presence of a TSG in this region (LYDIATT et al. 1994). Indeed, fine mapping of chromosome 11p in breast cancer suggests the presence of three potential TSG at 11p15 (NEGRINI et al. 1995). Furthermore, multiple regions of LOH on chromosome 11p have been identified in NSCLC (BEPLER and GARCIA-BLANCO 1994). The H-*ras* proto-oncogene is located at 11p15.5, and this region has been studied in detail by KIARIS et al. (1994, 1995) for H-*ras* mutations, overexpression by competitive reverse transcription (RT)-PCR and instability. Mutations in H-*ras* were not detected in any of the 120 SCCHN samples analysed, but 54% overexpressed this gene and five out of 14 samples exhibited 3′-VTR (variable tandem repeat) instability.

The long arm of chromosome 11 contains the MEN1 locus, associated with anterior pituitary insulinomas, at 11q12–q13. The 11q13 region also contains a number of proto-oncogenes, including *int-1*, *hst-1* and *bcl-1*/PRAD-1/cyclin D1. It is now recognised that the most likely candidate *bcl-1* gene is the PRAD-1/cyclin D1 proto-oncogene (HUNTER and PINES 1994). Allelic imbalance on chromosome 11q, at 11q13 or 11q23, has been demonstrated in 45% of SCCHN (AH-SEE et al. 1994). In cases of allelic imbalance, it can be difficult to distinguish between amplification and deletion of an allele. AH-SEE et al. (1994) concluded, following Southern blot analysis, that the imbalance found at 11q13 was largely due to deletion rather than amplification, suggesting the possible loss of a TSG at this site.

Amplification of 11q13, however, has been demonstrated in 30%–50% of SCCHN (BERENSON et al. 1989; SOMERS et al. 1990; LEONARD et al. 1991). The target gene of greatest prognostic significance at this locus appears to be PRAD-1/cyclin D1. There is compelling evidence that D-type cyclins are fundamental to cell cycle regulation (for a review, see HUNTER and PINES 1994). Two- to 12-fold amplification of the cyclin D1 gene has been demonstrated in 34%–37% of SCCHN (CALLENDER et al. 1994; JARES et al. 1994) and has been found to correlate with mRNA overexpression, aggressive or late-stage tumours, advanced local invasion and presence of lymph node metastases (CALLENDER et al. 1994; JARES et al. 1994). Overexpression of cyclin D1 has also been detected immunohistochemically in 63% of SCCHN and was found to correlate with a poor prognosis, being associated

with a more rapid and frequent recurrence of disease and shortened survival (MICHALIDES et al. 1995).

The 11q22–q24 region is commonly lost in breast cancer, and recently refined mapping has defined two independent areas of loss at 11q23 (NEGRINI et al. 1995). The finding of frequent loss at 11q23 in SCCHN (AH-SEE et al. 1994) raises the possibility of involvement of putative TSG in this region in the pathogenesis of head and neck tumours.

### 3.1.7 Chromosome 13

The RB TSG is located on the long arm of chromosome 13 at 13q14.2. Inactivation of the RB gene has been reported in a number of tumour types (other than retinoblastoma), including carcinomas of the oesophagus and lung (HUANG et al. 1993; REISSMANN et al. 1993). Detailed analysis of the long arm of chromosome 13 in 60 SCCHN using ten microsatellite markers has demonstrated LOH in at least one 13q marker in 52% of tumours (YOO et al. 1994). A high frequency of LOH was found at a marker mapping to 13q14.3, just telomeric to the RB gene. Function of the RB gene appeared to be largely unaffected, as the majority of SCCHN showed normal nuclear immunostaining for RB protein. Similar findings of allelic loss at 13q14 in the presence of RB protein expression have been reported in ovarian and breast cancer (BORG et al. 1992; DODSON et al. 1994); thus it is possible that another TSG is contained at 13q14. This is supported by the study carried out by SCHOLNICK et al. (1994), which found LOH at 13q14.3 in 59% of squamous cell carcinomas of the supraglottic larynx. Other regions of chromosome 13q showing LOH less frequently in SCCHN include 13q32 (LYDIATT et al. 1994).

### 3.1.8 Chromosome 17

The p53 TSG, contained on chromosome 17p, is commonly involved in the pathogenesis of human tumours, including SCCHN. Frequent LOH involving TP53, the p53 locus at 17p13.1, has been found in a number of studies of SCCHN (ADAMSON et al. 1994; NAWROZ et al. 1994; SCHOLNICK et al. 1994). Detailed study of chromosome 17 has shown LOH on 17p in 50% of SCCHN, often involving the TP53 locus (42%), but more frequently involving the CHRNB1 locus at 17p11.1–p12 (56%) (ADAMSON et al. 1994). Consideration of tumours from the hypopharynx alone increased LOH at CHRNB1 to 77%, strongly suggesting the presence of a TSG at this locus which is involved in the development of these carcinomas. Fine mapping with additional microsatellite markers is required to further define this region. In addition, 34% of SCCHN showed LOH on 17q (ADAMSON et al. 1994).

Mutations of p53 are frequent in many tumour types (HOLLSTEIN et al. 1991; GREENBLATT et al. 1994). Initial investigations of p53 expression in SCCHN demonstrated that approximately 60% of these tumours had immunohistochemically detectable p53, thus suggesting, but not necessarily indicating, the presence of p53 gene mutations (for a review, see FIELD et al. 1993). Immunostaining for p53 correlated with the patients' history of smoking (FIELD et al. 1991, 1994b).

Tumours from eight out of 12 non-smokers were p53 negative, whereas 36 out of 45 from heavy smokers were p53 positive. In addition, tumours from all but two of the 12 patients who had stopped smoking for more than 5 years prior to presentation immunostained for p53, suggesting that p53 gene alterations were an early event in the development of these cancers (FIELD et al. 1991, 1994b). Sequence analysis has recently confirmed the correlation between p53 mutations and heavy smoking (BRENNAN et al. 1995; LILOGLOU et al., unpublished). In addition to smoking, FIELD et al. (1994b) also correlated p53 data with the patients' history of alcohol consumption. A possible synergistic effect of these two "carcinogens" was demonstrated by logistic regression analysis ($p < 0.05$), thereby indicating that both smoking and alcohol consumption are probably linked to aberrant p53 expression in head and neck cancer. These findings have recently been corroborated by BRENNAN et al. (1995). The majority of molecular studies have now demonstrated that 40% or more of SCCHN contain p53 mutations (SOMERS et al. 1992; BOYLE et al. 1993; BRENNAN et al. 1995; for a review, see GREENBLATT et al. 1994). As the p53 TSG plays an important role in arresting the cell cycle to permit repair of damaged DNA and inducing apoptosis if damage is irreparable, loss of p53 function presumably results in genomic instability and the accumulation of genetic lesions. Other genes involved in maintaining genomic stability are discussed in Sect. 4.

Inactivation of p53 may also occur by non-genetic mechanisms, through interation with human papillomavirus (HPV) proteins. The E6 protein from high-risk HPV types, such as HPV-16, can complex with and facilitate the rapid degradation of p53 (WERNESS et al. 1990; SCHEFFNER et al. 1990; HUBBERT et al. 1992). Cells expressing HPV-16 E6 do not manifest a p53-mediated response to DNA damage (KESSIS et al. 1993). Presumably E6-mediated degradation of p53 abrogates its negative growth-regulatory effect and can result in genomic instability. In addition, HPV-16 E7 protein binds to the hypophosphorylated form of the RB TSG product (pRB) and may therefore prevent pRB-mediated suppression of cell growth (MÜNGER et al. 1989). High-risk HPV types have been detected in SCCHN, but prevalence figures vary greatly between studies; this variation may be due in part to different sensitivities of detection systems used (for a review, see SNIJDERS et al. 1994). A particular association between HPV-16/33 and tonsillar carcinomas, however, has been demonstrated (SNIJDERS et al. 1992). We have recently detected HPV-16 in 21% of SCCHN (excluding tonsillar carcinomas) by PCR, using HPV general and type-specific primers (SNIJDERS et al., 1996).

### 3.1.9 Chromosome 18

LOH on chromosome 18q has been reported in a range of cancers, many studies concentrating on the DCC (deleted in colorectal cancer) locus at 18q21.1. In a detailed study, ROWLEY et al. (1995) found LOH on 18q in 49% of SCCHN. The most frequent region of loss was 18q21.1–q21.3 using the marker D18S35 (33%), whereas loss at the DCC locus was found in only 12% of samples. Only one sample showed loss at both D18S35 and DCC, raising the possibility that another putative TSG may be located in this region (ROWLEY et al. 1995).

## 3.2 Allelotype Analysis of Squamous Cell Carcinoma of the Head and Neck – Fractional Allele Loss

Genetic changes at particular chromosome loci may occur as independent genetic events; they are, however, part of a complex tumour progression process. Allele losses are irreversible and will remain, and accumulate, as the cell proliferates. It may be that evaluation of the degree of genetic alteration or fractional allele loss (FAL), as determined by allelotype analysis, will provide useful molecular correlates of tumour behaviour.

FAL for a particular tumour is defined as the number of chromosomal arms on which allele loss is observed divided by the number of chromosomal arms for which allelic markers are informative (Vogelstein et al. 1989). In an allelotype of colorectal carcinomas, a positive correlation between high FAL and development of recurrent disease has been found. In addition, patients with a tumour containing high FAL were significantly more likely to die with or from their cancer (Vogelstein et al. 1989).

In the most comprehensive allelotype of SCCHN (Field et al. 1995b), 80 carcinoma samples were analysed using a total of 145 microsatellite markers on 39 chromosome arms (Fig. 3). FAL values were calculated for 52 of these carcinomas which had LOH information on nine to 39 chromosome arms (Fig. 4). This group of 52 tumours was composed of 36 previously untreated tumours and 16 previously treated tumours. The median FAL value for these tumours was 0.22 (mean, 0.25; range, 0.0–0.8), demonstrating that on average alleles were lost from 25% of

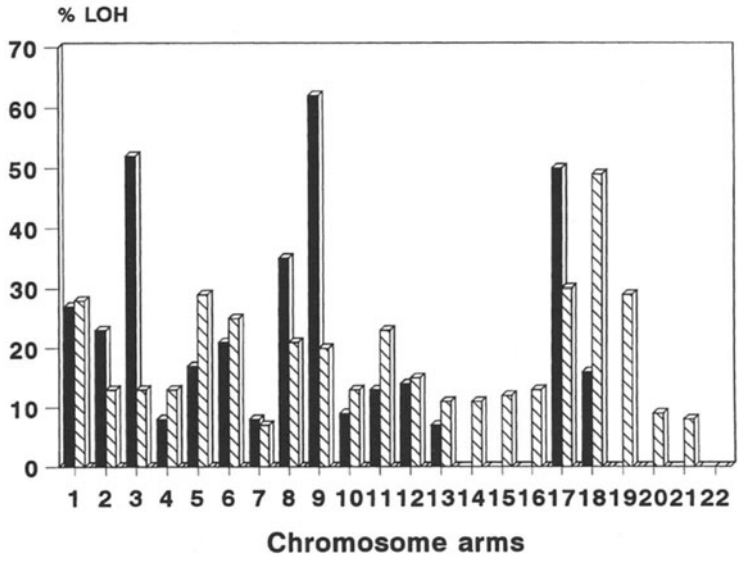


**Fig. 3.** Allelotype of squamous cell carcinoma of the head and neck. Frequency of allele loss on each chromosome arm in 80 carcinomas using 145 microsatellite markers. *Black bars*, p arm; *shaded bars*, q arm. *LOH*, loss of heterozygosity. (From Field et al. 1995b, with permission)

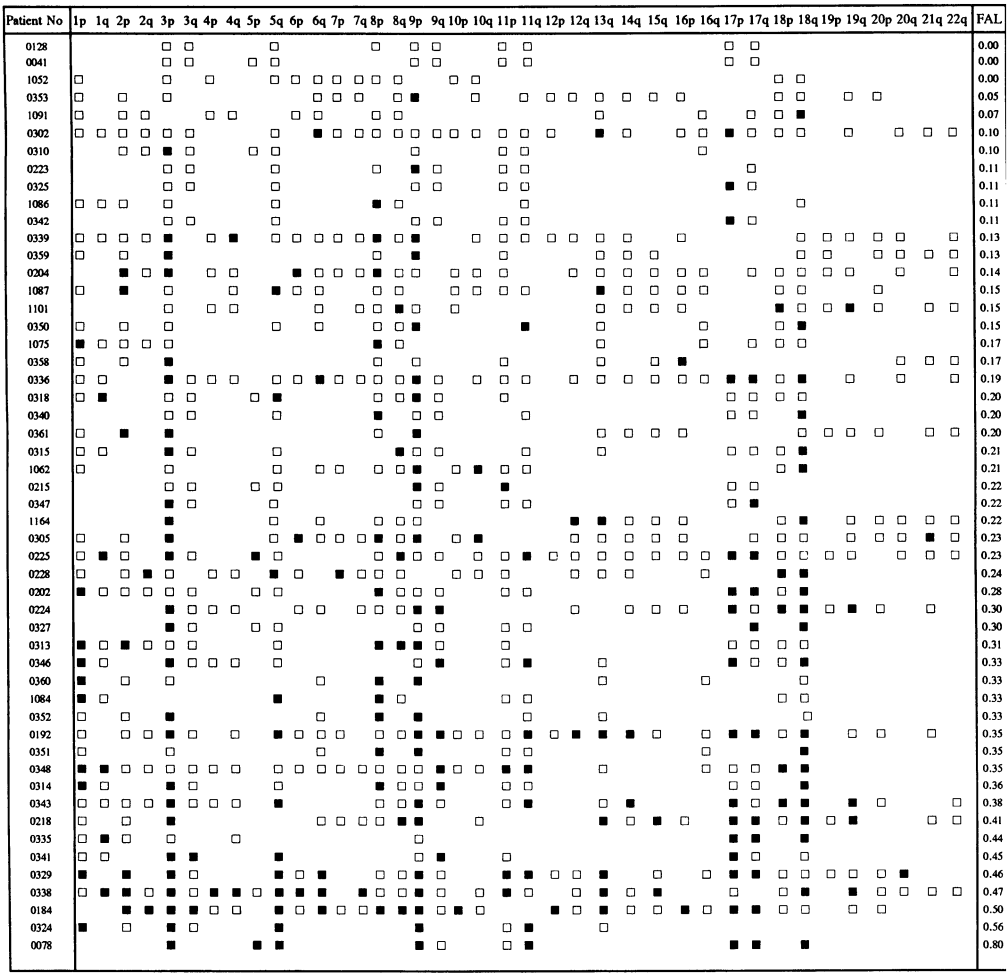


**Fig. 4.** Individual allelotypes for 52 squamous cell carcinomas of the head and neck examined on nine or more informative chromosome arms (range, nine to 36). Fractional allele loss (*FAL*) data is shown for each tumour sample. *Black boxes*, loss of heterozygosity (LOH); *white boxes*, retention of heterozygosity. Chromosome arms which were not informative or not examined are not shown. Each *square* represents the summation of LOH results on a single chromosome arm using all of the informative markers. (From FIELD et al. 1995b, with permission)

the chromosome arms examined. Tumours were divided into two subgroups – those with FAL greater than the median value and those with FAL less than the median value – and were correlated with clinico-pathological data (tumour site, tumour grade, TNM staging, nodes at pathology and patient history of smoking and drinking). A positive correlation was found between FAL and tumour grade ($p = 0.06$) and nodes at pathology ($p = 0.01$). The FAL data was also related to clinical outcome by log-rank analysis; FAL greater than the median value correlated with poor survival ($p < 0.032$), even when previously untreated tumours were analysed

separately ($p < 0.019$). Analysis of 40 advanced tumours (TNM III and IV) also demonstrated a correlation between FAL and prognosis. Thus accumulated genetic damage, as provided by allelotype analysis, provides a useful molecular indicator of the behaviour of head and neck carcinomas.

## 4 DNA Repair

The importance of DNA repair mechanisms in tumour avoidance has recently received much attention. Although the molecular processes involved in DNA repair have been studied in bacteria (*Escherichia coli*) and yeast (*Saccharomyces cerevisiae*) for many years, it is only recently that research has been focused on these processes in human cells. Fortunately, human cells use DNA repair mechanisms analogous to those in micro-organisms, and it is now recognised that a predisposition to certain cancers is caused by defects in these pathways.

### 4.1 Mismatch Repair and Familial Cancer

Insertion of an incorrect base or the addition of an extra nucleotide is a relatively common DNA biosynthetic error. Microsatellite repeat sequences are particularly susceptible to errors due to slipped-strand mispairing during replication (Kunkel 1993; Richards and Sutherland 1994). The role of the mismatch repair system is to recognise these mispairs and eliminate them from newly synthesised DNA strands.

Repair of mismatched DNA in *E. coli* requires a number of genes, including *mutS*, *mutL* and *mutH*. Similarly, studies with *S. cerevisiae* have identified three mismatch repair genes, a *mutS* homologue (MSH2) and two *mutL* homologues PMS1 and MLH1. Mutations in these genes result in a general elevation of spontaneous mutation rates and a dramatic destabilisation of microsatellite repeats (Modrich 1991; Strand et al. 1993).

The finding of widespread alterations in microsatellite repeats in familial colorectal cancer was a clue that defects in the human mismatch repair system might be involved. Hereditary non-polyposis colorectal cancer (HNPCC) is one of the most common familial cancer syndromes, characterised by early-onset carcinomas of the colon. In addition to colorectal cancer, a subset of affected individuals are also predisposed to endometrial, haematological, gastric, pancreatic, ovarian, skin and urinary tract cancers (Lynch et al. 1993). Tumours from HNPCC patients have been found to harbour frequent mutations within simple microsatellite repeat sequences, suggesting the occurrence of numerous replication errors during tumour development (Aaltonen et al. 1993; Ionov et al. 1993). Cell lines derived from these tumours display a mutator phenotype and can acquire mutations at rates more than 100-fold greater than that of normal cells (Parsons et al. 1993; Bhattacharyya et al. 1994; Eshleman et al. 1995). Four human mismatch repair genes have now been identified; the *h*MSH2 gene encodes

a protein homologue of bacterial *mutS*, while *h*MLSH1, *h*PMS1 and *h*PMS2 encode distinct *mutL* homologues (FISHEL et al. 1993; LEACH et al. 1993; BRONNER et al. 1994; NICOLAIDES et al. 1994; PAPADOPOULOS et al. 1994). The majority of HNPCC cases are attributable to a defect in any one of these four genes. Normal cells from the affected individual contain one functional and one defective copy of the involved repair gene, whereas in tumour cells the wild-type allele is inactivated by somatic mutation, resulting in defects in both copies of the affected gene (LEACH et al. 1993; NICOLAIDES et al. 1994). Thus it appears that genetic destabilisation in HNPCC tumours is due to the functional loss of critical mismatch repair activity.

## 4.2 Microsatellite Instability in Sporadic Cancers

If instability of microsatellite sequences is used as a marker of deficiency in human mismatch repair, it would appear that this mechanism is associated with the development of some non-familial cancers. Microsatellite instability (MI) has been demonstrated in sporadic cancers, including colon, breast, gastric, pancreatic, lung and endometrial carcinomas (HAN et al. 1993; RISINGER et al. 1993; THIBODEAU et al. 1993; RHYU et al. 1994; MERLO et al. 1994; SHRIDHAR et al. 1994; YEE et al. 1994). The stage at which MI occurs, however, appears to vary with the tumour type. Genomic instability is apparently an early event in colorectal cancer, as MI has been detected in adenomas (SHIBATA et al. 1994) and at similar frequency in ulcerative colitis-associated colorectal dysplasias and cancers (SUZUKI et al. 1994). MI has also been detected in early-stage breast cancer (YEE et al. 1994). In contrast, genomic instability appears to be a late event in gastric carcinomas, as MI has been detected significantly more frequently in poorly differentiated advanced-stage tumours than in well-differentiated early-stage tumours and has been infrequently detected in dysplastic tissue (HAN et al. 1993; CHONG et al. 1994; RHYU et al. 1994).

### 4.2.1 Squamous Cell Carcinoma of the Head and Neck

The presence of MI in SCCHN has recently been examined (FIELD et al. 1995a; Fig. 5). Fifty-six tumours were assessed using a total of 34 microsatellite markers on ten chromosomes; 25 of these tumours were examined with a minimum of ten (range, 10–34) microsatellite markers. Alteration observed in two or more markers was regarded as indicative of MI. Of the 25 SCCHN examined in detail, seven tumours (28%) were found to have MI in two or more microsatellite markers; three of these tumours contained alterations in at least 20 markers. The level of MI was assessed with clinico-pathological parameters and survival data in these 25 SCCHN patients. No correlations were found between MI and tumour site, previous treatment, degree of histological differentiation, nodal metastases, TNM stage or history of alcohol consumption. MI was detected in TNM stage I carcinomas, however, indicating that genomic instability may occur in the early stages of

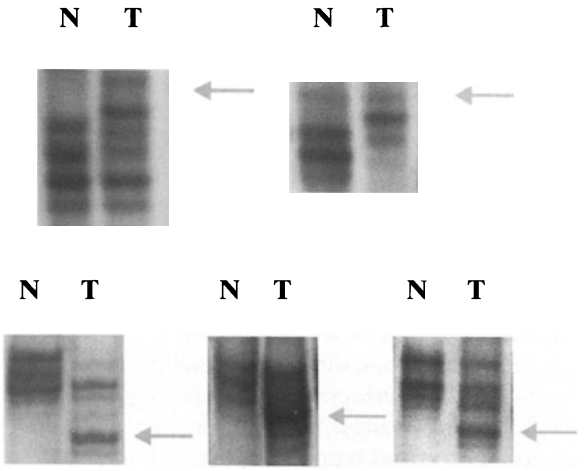


**Fig. 5.** Representative microsatellite instability detected in squamous cell carcinoma of the head and neck in patient no. 184 (*top*) and patient no. 224 (*bottom*). *Arrows* represent novel alleles. *N*, normal; *T*, tumour

tumour progression. There was a significant association between MI and smoking history, with instability being more frequent in tumours from non-smokers compared to smokers ($p = 0.02$). In a study of MI in SCCHN by Mao et al. (1994), a similar percentage (29%) of tumours to that found by Field et al. (1995a) exhibited microsatellite alterations with at least one marker.

Whether MI in SCCHN results from mutations in known mismatch repair genes is unknown. It is possible that alterations in other genes may be responsible, as many sporadic colorectal cancers which exhibit MI do not contain mutations in any of the four known human mismatch repair genes (Liu et al. 1995). We have previously reported that a history of heavy smoking correlates with overexpression of the p53 TSG (Field et al. 1991, 1992, 1994b), and these results are now supported by the demonstration of a significantly higher rate of p53 mutations in SCCHN from smokers compared with non-smokers (Liloglou et al., unpublished). The contrasting association of smoking history with MI and p53 mutations suggests that MI is a distinctive mechanism from that mediated by TSG in head and neck carcinogenesis. MI may occur relatively early in the progression of some SCCHN, particularly in non-smokers.

## 5 Conclusion

The characterisation of genetic aberrations in SCCHN by cytogenetic and allelic imbalance investigations has greatly increased our knowledge of the molecular mechanisms involved in the development of this disease. The cytogenetic studies of Jin et al. (1993, 1995) and Cowan (1992) and the three allelotype analyses (Ah-See et al. 1994; Nawroz et al. 1994; Field et al. 1995b) demonstrate the complexity

of the genetic mechanisms involved in the pathogenesis of SCCHN. Apart from the involvement of *ras* and *myc* oncogenes (FIELD et al. 1989; KIARIS et al. 1995) and the p53 TSG (FIELD et al. 1991), it is now apparent that alterations at many additional chromosome loci may also play a role in head and neck carcinogenesis (1p31.2–p21.3, 3p25, 3p21.3–p23, 3p13–p14, 5q21–q22, 8p21.2–p11, 9p21–23, 11p15.5, 11q13, 13q14, 17p11.1–p12, 18q21.1–q21.3; see Sect. 3). Fractional allele loss analysis has demonstrated that increased accumulation of this genetic damage correlates with a poor clinical outcome (FIELD et al. 1995b). The recent finding in SCCHN of MI, which has been associated with DNA repair defects in HNPCC, provides a completely new approach to investigating molecular mechanisms in cancer of the head and neck (MAO et al. 1994; FIELD et al. 1995a).

The correlation of specific genetic alterations with histopathological progression in colorectal cancer has permitted the development of a molecular model in which the accumulation of specific genetic changes is responsible for successive phases of clonal expansion (FEARON and VOGELSTEIN 1990; BOLAND et al. 1995). The development of such a model for head and neck cancer would have advantages in terms of early diagnosis, prognosis and management. From the data available to date, it would appear that mutation of the p53 TSG and LOH at 9p21–22 may be early events in the pathogenesis of SCCHN. p53 mutations have been demonstrated in pre-malignant lesions of the head and neck and increase in frequency with increasing severity of dysplasia and tumour progression (BOYLE et al. 1993; EL-NAGGAR et al. 1995b; SCHOLES et al., unpublished). LOH at 9p21–22 has been found at similar frequency in severely dysplastic lesions and carcinomas (VAN DER RIET et al. 1994). LOH on 9p has also been found in severe dysplasia by EL-NAGGAR et al. (1995a), but at a substantially lower frequency compared with carcinomas (28% and 72%, respectively). Amplification of the cyclin D1 gene appears to be a late event, correlating with advanced-stage tumours and metastases (CALLENDER et al. 1994; JARES et al. 1994). Overexpression of the c-*myc* oncogene has been associated with a poor prognosis (FIELD et al. 1989), whereas *ras* overexpression has been correlated with a favourable prognosis (KIARIS et al. 1995).

It is important to recognise that the genetic alterations which occur during tumour progression in smokers may differ from those in non-smokers. Our studies have shown that immunohistochemical detection of p53 in SCCHN correlates with a history of heavy smoking (FIELD et al. 1991, 1992, 1994b). In addition, we have found a significantly higher prevalence of p53 mutations by sequence analysis in SCCHN from smokers compared with non-smokers (LILOGLOU et al., unpublished). In contrast, MI is more common in SCCHN from non-smokers than smokers (FIELD et al. 1995a), as are multiple allelic deletions (LYDIATT et al. 1994). It is of interest that *ras* mutations are uncommon in head and neck carcinomas from Europeans (KIARIS et al. 1995), but are frequently found in oral tumours in India (SARANATH et al. 1991); the differing results in Indian subjects may be related to their habit of chewing tobacco.

Further characterisation of the genetic changes involved in head and neck carcinogenesis is imperative for improvements in the diagnosis, prognosis and therapy of these tumours. Of particular importance is the investigation of pre-malignant lesions, the malignant potential of which cannot be determined by

conventional histopathological methods. Elucidation of the specific genetic alterations that are important in the initial stages of carcinogenesis will be critical in predicting which of these lesions progress to malignancy, thereby allowing early intervention.

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# Oncogenes and Growth Factor Receptors As Diagnostic and Prognostic Markers in Precancers and Cancers of the Oral Mucosa*

A. BURKHARDT



## 1 Introduction

Cancer, if not in essence a genetic disease, is at least to a large extent determined in its behaviour by the interaction of a number of genetically coded products, which may enhance its growth (oncogenes) or suppress it (tumour suppressor genes or anti-oncogenes). However, the genetic changes of various cancers show considerable individual variations some of which maybe determined by geographic or aetiological factors. Overall, 50%–96% of oral carcinomas have abnormal karyotypes (OWEN et al. 1992; HITTELMAN et al. 1993), and there is a high frequency of breakpoints and deletions (OWEN et al. 1992; PATEL et al. 1993; PARTRIDGE et al. 1994; FÜZESI et al. 1994). All chromosomes may be involved, and it is noteworthy that those chromosomes encoding some of the well-known oncogenes (or tumour suppressor genes) are frequently altered, such as chromosome 1, 3, 7 (epidermal growth factor receptor, EGFR), 8 (*myc*), 9 (MTS), 11 (*ras*, *bcl*1, *int*1), 13 (*erb*), 17 (*neu*, p53) and 18 (PATEL et al. 1993; VORAVUD 1993; TSUJI et al. 1994). Details are outlined in the chapter by Scholes and Field in this volume.

Proto-oncogenes are present in all cells, and in humans some 40 different types are known. They code for proteins which normally have important functions in growth regulations and differentiation of cells and tissues such as growth factors, growth factor receptors, and gene expression regulators. Especially in embryogenesis, many of these genes are temporarily expressed. Also in normal and altered tissue with deregulated growth, these genes may under certain circum-

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stances (for example, by transduction by retroviruses or physical/chemical factors) be rearranged, activated, overexpressed or mutated and become oncogenes. Activation of oncogenes leads to an enhanced cellular growth potential, and some growth receptors may be activated by false signals; mutation of antioncogenes may result in loss of function and thus has the same effect as oncogene overexpression. Oncogenes may induce abnormal growth factors or growth factor receptors. Oncogene tumour suppressor gene alterations maybe instrumental in tumour initiation, but most such changes are acquired during the promotion phase of carcinogenesis or even later during tumour progression. Thus precancerous lesions or early carcinomas as a rule exhibit fewer changes of oncogene/tumour suppressor gene expression than do progressed tumours. On the other hand, the extent of oncogene activation or tumour suppressor mutation without question influences tumour behaviour and may thus be of prognostic value for established carcinomas. In lesions considered potentially premalignant, determination of these changes maybe instrumental in differentiating true premalignant precancerous lesions from harmless aberrations like hyperplasia.

## 2 Methodological Problems

The demonstration of oncogene/tumour suppressor gene alterations in normal tissues and in tumours may be achieved by molecular biochemical methods on tissue homogenates or by specific staining of tissue sections (in situ demonstration). Both methods may show either the specific DNA/RNA sequences of the oncogene/tumour suppressor gene or the gene products, i.e. the proteins. Single-stranded nucleic acids have a tendency to form double-stranded helices (hybrids) with nucleotides in complementary sequence. Using labelled (radioactive isotopes, biotinylated sequences) nucleic acids makes it possible to demonstrate specific DNA/RNA sequences by hybridisation in tissue homogenates. The sensitivity maybe greatly enhanced by polymerase chain reaction (PCR). In situ localization maybe achieved by in situ hybridisation or immunohistochemical staining of the gene product proteins. Certainly the biochemical method of determination is more sensible, may be suitable for exact quantification, is generally more specific, and is able to identify even minor mutations. However, the specificity makes it very sensitive to contamination (e.g. traces of DNA) and other methodological faults; thus single publications without confirmation by other groups should be regarded sceptically. Also it must be kept in mind that mere oncogene expression is a widerspread phenomenon in tissues of different histogenesis, and quantitative analysis is necessary prior to propagating it as being of diagnostic or prognostic relevance (RIVIÈRE et al. 1990). Furthermore, examining a tissue homogenate never provides conclusive evidence of the cell fraction the substance identified is derived from, and of how high the concentrations in the tumour cells actually were. Tissue samples may contain less than 10% vital tumour cells.

In situ hybridisation and immunohistochemistry have the advantage of localising the positive signal, even to a specific cellular compartment (nucleus, cytoplasm, cell membrane). However sensitivity is low compared with that of

biochemical methods as a rule, not allowing, for example, distinction of mutant and non-mutant protein, and hybridisation may give false-positive results due to the occurrence of intervening repetitive sequences of nucleic acids. Also quantification is only roughly possible. For practical reasons, positive histochemical staining either by hybridisation or immunotechnique is considered to mark amplification or overexpression of the gene under consideration.

Most biochemical examinations of oncogenes/tumour suppressor genes have been applied to larger solid tumours, especially breast carcinomas and colonic carcinomas. A larger tumour mass makes it possible to select an area with a high percentage of vital tumour cells and little tumour stroma. This limits our knowledge of oncogene expression to progressed tumours with many secondary genetic changes. However, the more interesting early alterations seem to be of greater interest, as they may give insight into aetiological and prognostic factors. Precise correlation to histomorphology is impossible in tissues composed of heterogeneous cell populations, so that the assessment of precancerous lesions by this method is limited. In extensive multifocal lesions (e.g. in the mammary ductal system), a higher percentage of atypical cells may be harvested in tissue blocs; in lesions of superficial covering epithelium with only focal atypias no meaningful results can be expected.

A new method combining the histomorphological localisation with molecular genetic analysis by microdissection of paraffin-embedded tissue is promising to bridge this gap. By this method, YOUNGSON et al. (1995) were able to demonstrate that a single copy of HER 2/neu identified by molecular gene analysis in breast carcinomas or normal tissue resulted in a negative immunostaining, while fourfold to eightfold amplification was detected by positive immunostaining, thus confirming conventional wisdom.

Unfortunately, there are very little reliable data on gene expression in oral carcinomas and even less for oral precancerous lesions (reviewed in BURKHARDT 1985; TALACKO et al. 1991; FIELD 1991, 1992; SCULLY and BURKHARDT 1993; SCULLY 1993; BRACHMAN 1994). The meaning of these factors for diagnostic and prognostic assessment of cancerous and precancerous oral lesions is thus still very limited. In any case it seems of the utmost importance to distinguish results obtained by molecular genetic studies from in-situ demonstration on the one hand and results on progressed and early or precancerous lesions on the other.

## 3 Oncogenes

### 3.1 Growth Factor and Growth Factor Receptor-Related Genes

Epidermal growth factor (EGF) a polypeptide of 6000 daltons, stimulates the replication and also differentiation of a number of different cell types (CASTELLANI et al. 1994). EGF is counteracted by inhibitory growth regulators – so-called chalones, probably also polypeptides – which have not yet been defined in the oral mucosa (IVERSEN 1985). EGF, probably of fibroblastic origin

(SHIRASUNA et al. 1991) is normally found in small amounts in the subepithelial region of the oral mucosa, but is not expressed by epithelial cells. It is increased in dysplastic and malignant epithelial proliferations (SHIRASUNA et al. 1991). The fact that also in these circumstances the epithelium does not express EGF seems to indicate that the autocrine hypothesis (SPORN and TODARO 1980), i.e. permanent stimulation of tumour cells by self-secreted growth factor, does not apply to oral carcinomas. In squamous cell carcinoma cell lines, EGF even has a growth inhibitory function (KAMATA and ENOMOTO 1994).

Fibroblast growth factors (FGF 1 and FGF 2) on the other hand have been shown to be expressed by cells of oral squamous cell carcinomas in high frequency and enhanced intensity as compared to normal oral epithelium, and it has been speculated that these growth factors may contribute to cancer cell growth (MYOKEN et al. 1994). Also transforming growth factor-alpha (TGF-$\alpha$) is produced by squamous cells and squamous cell carcinoma cells, and there may be EGFR/TGF-$\alpha$ autocrine stimulation in these carcinomas (REISS et al. 1991; WONG 1993). Eosinophils may be a further source of TGF-$\alpha$ (WONG 1993). TGF-$\alpha$ is increased in oral leukoplakias compared with normal mucosa and decreases during treatment with retinoids, suggesting that it maybe an intermediate endpoint in cancer chemoprevention (BEENKEN et al. 1994). Human oral carcinoma cell lines produce more TGF-$\alpha$ than normal keratinozytes, but presence of a neutralising antibody in conditioned medium failed to produce a decrease in cell replication (PRIME et al. 1994a), casting doubt on the importance of this mechanism. Transforming growth factor-beta (TGF-$\beta$) is produced by normal and malignant keratinocytes and inhibits epithelial replication. Malignant cells may become refractory to this inhibition (PRIME et al. 1994b).

EGF and TGF-$\alpha$ act by binding to a specific receptor (EGFR). The EGFR is found on many cell lines, but especially on proliferating epithelial cells. Normally it is found in high concentration on basal cells of stratified squamous epithelium of skin and mucous membranes (CHRISTENSEN et al. 1992a). It is a 170kDa transmembranous (COHAN et al. 1982) glycoprotein with tyrosine kinase activity (DOWNWARD et al. 1984a). It consists of an outer portion, which binds EGF and TGF-$\alpha$, a transmembranous portion, and a cytoplasmic portion which contains the tyrosine kinase. DOWNWARD et al. (1984b) in a study on the Avian erythroblastose virus (AV) found a significant homology of the cytoplasmic portion of the EGFR and the v-*erb* B oncoprotein sequence. The EGFR has been shown to be a product of the c-*erb*B-1 protooncogene. The observation that the ligand enhances cellular replication and that during carcinogenesis changes in the receptor are found have led to the proposition that overexpression of the receptor in a number of neoplasms is paramount to their growth potential and thus its aggressiveness.

The prognostic implication of the expression of EGFR in tumours, especially mammary, gastric, cervical, and urinary bladder carcinomas has been intensively investigated. A number of studies in patients with breast carcinomas showed that patients with EGFR-positive tumours had a reduced recurrence-free survival and a shorter overall survival time than patients with EGFR-negative tumours (SAINSBURY et al. 1985a,b, 1987; COSTA et al. 1988; LEWIS et al. 1990), although other studies could not show significant differences (FOEKENS et al. 1990; SPYRATOS et al. 1990).

In the oral mucosa, EGFR expression has been reported in potentially premalignant lesions such as leukoplakias and preferentially in well-differentiated squamous cell carcinomas and cell lines derived from them (YAMAMOTO et al. 1986; EISBRUCH et al. 1987; WONG 1987; WONG and BISWAS 1988; PARTRIDGE et al. 1988; WEICHSELBAUM et al. 1989; ISHITOYA et al. 1989; OH et al. 1989; TODD et al. 1989; EL-ZAYAT et al. 1991; KIM et al. 1991; SHIRASUNA et al. 1991; YAMADA et al. 1992). In leukoplakia, EGFR expression has been found to be increased compared with normal mucosa (BEENKEN et al. 1994). In dysplasias of the oral mucosa, CHRISTENSEN et al. 1992a noted an extension of the EGFR-positive cells from the normally positive basal cell layer to all cells and cell layers.

SAKAI et al. (1990) could demonstrate EGFR by immunohistochemistry in four of 28 cases of oral carcinomas with a location mainly in the cytoplasm, but seldom on the cell surface; a strong expression was observed in mitotic cells. PARTRIDGE et al. (1988) studied 20 oral carcinomas and found variations of receptor expression, but no correlation to prognosis. ISHITOYA et al. (1989) reported overexpression in 53% of 15 carcinomas, SAKAI et al. (1990) reported the same in four of 28 cases. TALACKO et al. (1991), in a study of 35 oral squamous cell carcinomas, noted an extremely heterogeneous expression of EGFR, but noted that poorly differentiated carcinomas had a tendency to express high levels especially in the proliferating "basal" portion of the tumour. Also CHRISTENSEN et al. (1992a,b) found a varied distribution and staining pattern and stronger expression in poorly differentiated carcinomas with staining of all tumour cells in 40 oral and 15 laryngeal squamous cell carcinomas. A study on laryngeal carcinomas confirms the association of EGFR expression and poor tumour differentiation (SCAMBIA et al. 1991), while other studies on head and neck squamous cell carcinomas could not confirm this relationship (SANTINI et al. 1991). SARANATH et al. (1992) observed amplification and overexpression of the EGFR gene in 25% of 84 primary oropharyngeal cancers, while RIKIMARU et al. (1992), in a study of four cases, noted that EGF binding capacity of the tumour cells does not always parallel amplification of the EGFR gene.

YAMADA et al. (1992) report that, of 47 oral carcinomas, 51% contained EGFR-positive cells as determined by immunohistochemistry, and one of 25 cases exhibited fourfold gene amplification on molecular genetic analysis. In contrast to most authors, they report an association of EGFR expression with well-differentiated carcinomas. Some clinical studies indicate that, in oral lesions too, high expression of EGFR is associated with a poor prognosis (OZANNE et al. 1986; HENDLER et al. 1989). OHI et al. (1993) in a study of 68 patients with squamous cell carcinomas found positive reactions in all tumours and noted that 28 of 36 patients in whom staining was stronger than that in the normal cells, but only 12 of 32 patients in whom the staining intensity was equal to or weaker than that in basal cells had lymph node metastasis. In vitro studies showed that overexpression of EGFR is not an invariable characteristic of human oral squamous carcinoma-derived cell lines (PRIME et al. 1994a). In experimental carcinogenesis in the hamster cheek pouch using inoculation of dimethyl-benzanthracene (DMBA) or DMBA plus herpes simplex virus (HSV)-1, amplification and overexpression of c-*erb* B 1 has been observed (OH et al. 1989).

The avian erythroblastosis virus encodes a v-erb B protein which is a crippled EGFR lacking the EGF binding site. It is similar to the *neu* oncogene initially identified in rat neuroblastomas. In humans, the equivalent is c-*erb* B 2 or HER 2 (human *erb*-B related) gene on chromosome 17. The product of the c-*erb* B 2 is a membrane-bound 185kDa receptor protein with tyrosine kinase activity also termed p185. It exhibits amino acid sequence homology to the EGFR encoded by the c-*erb* B 1 gene. C-*erb* B 2 has been found to be frequently amplified or overexpressed in adenocarcinomas, e.g. of the breast, stomach, kidneys, ovaries and pancreas.

SLAMON et al. (1987) investigated the prognostic relevance of the c-*erb* B 2 oncogene in breast carcinomas and found that overexpression was correlated with shorter recurrence-free survival and shorter overall survival of the patients. These results have been confirmed by a number of authors (TANDON et al. 1989; WALKER et al. 1989; WRIGHT et al. 1989; BORG et al. 1990; BORRESEN et al. 1990; PAIK et al. 1990; TOIKKANEN et al. 1992; for review, see ANDERSON 1992).

Overexpression of c-*erb* B 2 was not observed in hyperplastic or dysplastic intraductal lesions of the mammary gland, but in 56%–77% of preinvasive intraductal carcinomata in situ and most often in those lesions associated with invasive carcinoma (ALLRED et al. 1992).

C-*erb* B 2 has been demonstrated in basal cells of oral mucosa (GULLICK et al. 1987). A number of molecular genetic studies could not demonstrate amplification of c-*erb* B 2 in oral or oropharyngeal cancers (EASTY et al. 1986; RIVIÈRE et al. 1990; MERRITT et al. 1990; SOMERS et al. 1990; TALACKO et al. 1991; LEONARD et al. 1991; FIELD 1992). Amplification, overexpression and rearrangements of the gene in human oral squamous carcinomas were observed by LI et al. (1992) by DNA und RNA dot blot hybridisation. Transcription of c-*erb* B 2 could be detected in normal squamous epithelium and in oral carcinomas by RIVIÈRE et al. (1990). In constrast to molecular studies, immunohistochemically positive staining for the protein may be obtained in premalignant and malignant oral mucosal lesions.

BEEKEN et al. (1994) observed increased staining of oral leukoplakias as compared with normal mucosa. In a spectrum of 86 specimens from normal, hyperplastic, dysplastic and malignant human oral mucosa, HOU et al. (1992) noted a progressive increase in c-*erb* B 2 expression. In oral squamous cell carcinomas, FIELD et al. (1992) demonstrated a positive cytoplasmatic staining in 60% of 75 specimens, CRAVEN et al. (1992) in contrast found cell surface staining in 41% of 93 specimens. Both found no correlation to tumour differentiation, stage or survival. In our own experience, expression of EGFR and c-*erb* B 2 in precancerous oral lesions and carcinomas may be very inconsistent and in contrast to breast carcinomas may not yet be considered as a standard diagnostic procedure. Correlations with prognosis have to be considered with caution.

## 3.2 Guanosine Triphosphate Binding Oncogenes

The group of guanosine triphosphate (GTP) binding oncogenes is represented by the so-called *ras* gene family on chromosome 11 (Harvey-*ras*, Kirsten-*ras*, N-*ras*)

of three closely related genes, which encode a 21 kDa protein (ras p21) localized on the inner side of the plasma membrane with exhibition of GTPase activity. The mutant p21 probably binds to GTP or guanosine diphosphate (GDP), resulting in reduced enzymatic degradation thus creating a prolonged signal for cell proliferation. Such mutations have been demonstrated to be instrumental in the initiation and promotion of chemical carcinogenesis and are found in a number of neoplasias notably in bladder carcinomas and also in carcinomas of the breast, pancreas, stomach, lung, uterus, thyroid and liver (GULBIS and GALAND 1993). The importance of the *ras* oncogenes in oral precancerous and malignant lesions has not been fully elucidated.

In experimental DMBA-induced cancers amplification of H-*ras* or N-*ras* was found (WONG and BISWAS 1988; SHIN et al. 1993); in carcinomas induced in mice by 4-nitroquinoline 1-oxide, H-*ras* mutations were detected in ten of 14 invasive carcinomas, two of four carcinomata in situ, one of five dysplastic lesions and in none of two normal tissues (YUAN et al. 1994). In oral squamous papillomas, *ras* mutations were found in ten of 24 specimens in the spinous layer (SATOH et al. 1990.) ANDERSON et al. (1994) discuss a strong association between human papilloma virus (HPV) infection and activation of the H-*ras* gene in oral verrucous carcinoma. In their material, 6% of 27 oral squamous cell carcinomas demonstrated point mutations in the H-*ras* gene, and three of these contained both HPV-DNA and H-*ras* gene point mutations.

The study by FIELD and SPANDIDOS in 1987 gave first hints of an involvement of the *ras* mutations in oral squamous cell carcinomas, as all 14 oral squamous cell carcinomas of their series exhibited significantly higher expression of H-*ras* and K-*ras*. In the study by HOWELL et al. (1989), four of five patients with oral squamaous cell carcinomas were constitutionally heterozygous at the c-H-*ras* 1 locus, and the tumour had lost heterozygosity in one case.

A number of publications have reported various involvement of the ras mutations in oral precancerous lesions and carcinomas (AZUMA et al. 1987; SHENG et al. 1990; MERRITT et al. 1990; RUMSBY et al. 1990; FREER et al. 1990).

KANNAN et al. (1994) studied the expression of *ras* p21 by immunohistochemistry in normal, premalignant and malignant lesions of the oral mucosa. In normal keratinizing mucosa, expression was found in the basal and lower spinal cells, while non-keratinizing epithelium was negative. All other lesions showed more or less similar expression patterns, mostly confined to the basal or basaloid cells of leukoplakia or carcinoma and an increase with malignancy. The occurrence of *ras* p21 was often parallelled by positive EGFR expression, suggesting a relationship of both to cell proliferation.

HOWELL et al. (1990) found K-*ras* overexpression in only one of 11 oral carcinomas, and YEUDALL et al. (1993) demonstrated mutant H-*ras* gene in only one of 12 oral carcinomas. Some studies revealed that there are apparently important geographic differences concerning *ras* gene changes in oral carcinomas which again may reflect differences in etiology. RUMSBY et al. (1990), CHANG et al. (1991), and WARNAKULASURIYA et al. (1992) reported H-*ras* mutations to be infrequent in oral squamous cell carcinomas among British white caucassian populations, while SARANATH et al. (1989) found N-*ras* amplification in seven and K-*ras* amplification in 23 of 23 oral cancers associated with tobacco chewing in India and in a

subsequent study H-*ras* mutations in 20 of 57 tumours also in India (SARANATH et al. 1991). Studies on H-*ras* 1 restriction fragment length polymorphism (RFLP) by BHOITE et al. (1993) revealed that the heterozygous genotype occurred more frequently in normal individuals in India (53%) than in cancer patients (36%), thus limiting the utility of H-*ras* RFLP as a genetic marker for cancer risk.

In a study from Taiwan, six of 33 (18%) tumour specimens from betel quid chewers contained K-*ras* codon 12 mutations and four contained more than one mutation (KUO et al. 1994). In Japan, expression of *ras* p21 product was detected in 44 of 67 specimens (65.7%) of oral cancer, with the highest incidence in patients in the fifth decade and in tumours of the buccal mucosa (SATOH et al. 1992). HOELLERING and SHULER (1989) detected H-*ras* mRNA and p21 in all five oral squamous cell carcinomas examined and noted a non-uniform distribution with high expression in areas of high proliferation and also in dysplastic epithelium. In a study in Belgrade by MILASIN et al. (1994) five of nine specimens (55%) of carcinomas of the lip vermilion harboured mutations, four in codon 12 and one in codon 13.

In the United States, an immunohistological study by MCDONALD et al. (1994) revealed that 15 of 22 primary carcinomas (68%) stained positive for H-*ras*, ten for K-*ras* (45%), and seven for N-*ras* (32%). This was associated with increased tumour size and later stages of disease, but there was no correlation to lymph node involvement, site, differentiation, sex, age or race. The authors conclude that overexpression of the *ras* gene family occurs as a relatively late, but important event in oral squamous cell carcinoma. Two Japanese studies also associate *ras* expression to poor prognosis (AZUMA et al. 1987; TSUJI et al. 1989). In contrast, FIELD (1992) reports that overexpression of the p21 *ras* in squamous cell carcinomas correlates with favourable prognosis in the disease-free group of cancer patients and conclude that it may be an important event in early stages of cancer. The relationship of *ras* mutation to etiology (tobacco, chewing habits), stage of disease and geographical distribution certainly awaits further clarification.

### 3.3 Nuclear Protein-Related Oncogenes

The members of the so-called *myc* oncogene family (c-*myc*, l-*myc* and n-*myc*) code for a nuclear protein of 62 kDa and are involved in DNA binding and regulation of transcription, activating cell replication and influencing differentiation and apoptosis. Especially amplification and elevated expression of c-*myc* (on chromosome 8) are found in a number of malignant lymphomas and solid tumours, e.g. small-cell carcinomas of lung, breast and ovarian carcinomas, and are as a rule associated with poor prognosis.

C-*myc* overexpression could be demonstrated in all of 14 squamous cell carcinomas of the head and neck (FIELD and SPANDIDOS 1987; FIELD 1991). Most subsequent studies confirmed overexpression with or without amplification of the c-*myc* gene in oral carcinomas (YOKOTA et al. 1986; VOLLING et al. 1988; BERENSON et al. 1989; SARANATH et al. 1989; LEONARD et al. 1991).

SAKAI et al. (1990) in an immunohistological study found positive staining of all of 27 oral carcinomas for the c-*myc* oncogene product and noticed three staining patterns: nuclear staining, perinuclear staining, and diffuse cytoplasmic staining. A pronounced staining was seen in mitotic cells.

Ten of 14 oral squamous cell carcinomas associated with tobacco chewing showed molecular lesions in *myc* genes (SARANATH et al. 1994b). Two of eight carcinomas in the series examined by HAUGHEY et al. (1992) exhibited increased c-*myc* copy numbers, and amplification may also be found in metastatic tumour tissue. This is considered to indicate an increased metastatic potential of tumour cells with c-*myc* amplification (HAUGHEY et al. 1992).

Oral cancer cell lines may also show overexpression of c-*myc* mRNA (INAGAKI et al. 1994). RIVIÈRE et al. (1990) could demonstrate transcription of c-*myc* in normal tongue mucosa and primary and metastatic oral squamous cell carcinomas. In oral papillomas, the c-*myc* oncogene product was demonstrated by immunohistochemistry in 17 of 25 specimens (70.8%) and was located mainly in basal cells (SATOH et al. 1992). In a study of oral precancerous and early invasive oral lesions, c-*myc* nuclear labelling correlated with progressive histological atypia (EVERSOLE and SAPP 1993), but some hyperkeratotic lesions without dysplasia were positive for the c-*myc* oncoproteins, possibly labelling those few cases of non-dysplastic leukoplakias transforming into carcinoma that are not detected by conventional assessment of dysplasia. Most studies link c-*myc* amplification or overexpression to advanced stages of oral carcinomas and correlate it with tumour progression and poor prognosis (YOKOTA et al. 1986; FIELD et al. 1986, 1989; BUTT et al. 1990; FIELD 1991). FIELD et al. (1989) in a study of 44 squamous cell carcinomas of the head and neck found c-*myc* expression in 21 cases (48%), and decreased survival was documented for these patients. No correlation could be established to age, sex, tumour stage, lymph node metastasis of differentiation in this study.

An immunohistochemical study of 30 tumours with 11 strongly positive for c-*myc* (37%), five moderately positive, and 14 (47%) negative (GAPANY et al. 1992), revealed a correlation between negative c-*myc* reaction and number of metastatic nodes. The authors speculate that loss of c-*myc* oncoprotein might be associated with aggressive behaviour of squamous cell carcinomas.

For c-*myc* oncogene too, further studies are necessary for establishing a clear link to prognostic assessment. L-*myc* may be increased in squamous carcinoma cell lines (YIN et al. 1991). N-*myc* alterations may be frequently present in normal healthy individuals and in oral cancer patients in India (SARANATH et al. 1994a).

## 3.4 Protein Kinases and Other Oncogenes

A number of membrane-associated protein kinases may function as proto-oncogenes and oncogenes. Of these the oncogenes *abl*, *fes*, *mos* and *raf* have been investigated in oral carcinomas. None was significantly amplified or overexpressed (SPANDIDOS et al. 1985; FIELD and SPANDIDOS 1987; SARANATH et al. 1989; MERRITT et al. 1990; LEONARD et al. 1991; FIELD 1992). Of the various oncogenes

without a defined mechanism of action, *int*-1, *int*-2 (Somers et al. 1990), *hst*-1 (Muller et al. 1994) and *bcl*-1 (Berenson et al. 1989) and *bcl*-2 have been demonstrated to be increased in head and neck carcinomas. The *bcl*-1 and -2 oncogenes inhibit apoptosis (programmed cell death) and may delay terminal differentiation in epithelial cells with subsequent hyperkeratosis (Dawson et al. 1995). *Bcl*-1 amplification was found in eight of 23 head and neck carcinomas, and this was seen more often in poorly differentiated squamous carcinomas and may be associated with poor prognosis (Berenson et al. 1989).

Chromosome 11q13 (which contains *bcl*-1) amplification is common in oral squamous cell carcinomas (Jin et al. 1990; Muller et al. 1994; Gaffey et al. 1995). The same region contains several other putative oncogenes such as cyclin D 1 (PRAD 1, CCND 1), *hst*-1, *int*-2, EMSI, and the gene for the drug detoxifying enzyme glutathione-*S*-transferase-pi (GST-pi) (Gaffey et al. 1995). Gaffey et al. 1995 examined squamous cell carcinomas of the head and neck, both by Southern blot hybridisation and immunohistochemistry. Anticyclin D 1 labelled 44% of the tumours (with a prevalence of hypopharyngeal cancer), while cytoplasmatic immunoreactivity for GST-pi was found in 85%. Twenty-four tumours showed twofold to tenfold amplification of 11q13 loci, two of these were coamplified for GST-pi. No correlations to grade of malignancy, invasive pattern, stage, or survival was noticed.

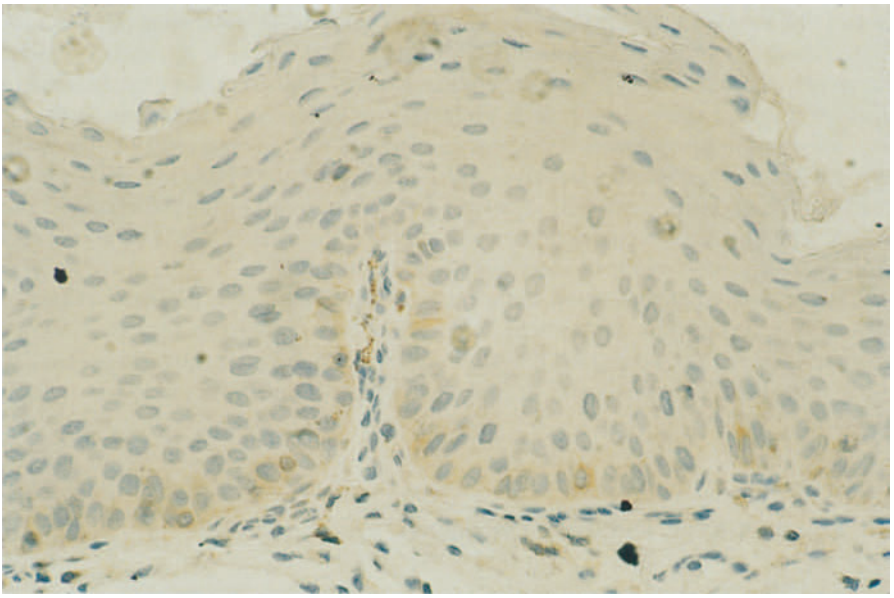

**Fig. 1.** Bcl-2 expression confined to single cells of the basal cell layer of a hyperplastic oral mucosal lesion (clinical manifestation: leukoplakia of the floor of the mouth in a 55-year-old male patient). There is weak positive staining of the cytoplasm of the basal cells indicating the presence of the 25 kDa protein within the mitochondria. This pattern of expression is found for the products of a number of oncogenes and suppressor genes

The *bcl*-2 encodes a *bcl*-2 protein (BCLP) which is located in mitochondria and is regularly found in centrocytic malignant lymphomas. In oral mucosa, normal basal cells are negative for BCLP, but in hyperplastic lesions like leukoplakia it maybe expressed (Fig. 1).

## 4 Tumour Suppressor Genes

Of the various known tumour suppressor genes, p53 has been most intensively investigated in tumours of various organs and in oral lesions. In view of its importance it is separately treated in the chapter by Slootweg (this volume). Further tumour suppressor genes, Rb and DCC, have been described in oral cancer cell lines (KIM et al. 1993). The MTS 1/CDK 41 gene on chromosome 9p21 is supposed to encode a 16kDa cyclin kinase inhibitor that is instrumental in tumour suppression. It was found to be mutated in cell lines derived from primary and metastatic oral squamous cell carcinomas (YEUDALL et al. 1994). A correlation of expression of a putative metastasis suppressor gene, the nm 23-2/NDP kinase alpha gene, with the metastatic potential of metastatic clones from a spontaneous rat oral squamous cell carcinoma could not be established (HENDERSON 1993).

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